THE DEVELOPMENT OF BOVINE PREANTRAL FOLLICLES *IN VITRO*

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Ph.D.

UNIVERSITY OF EDINBURGH

2001

Declaration

I declare that this degree has been composed by myself and has not been submitted for any previous degree. The work described herein is my own and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the course of these studies.

Abstract

Satisfactory development of preantral follicles from humans and domestic ruminants *in vitro* remains elusive. The aims of this thesis were to use a serum-free culture system to identify regulators of early follicle and oocyte development. Preliminary experiments determined that bovine preantral follicles grow and produce increasing amounts of oestradiol throughout a six day culture period. Neither FSH nor IGF-1 significantly increased follicle diameter. However, FSH did promote follicular oestradiol secretion. The dissociation of follicular growth from steroidogenenic function indicated that measurement of follicular diameter may not be a reliable marker of physiological follicular development *in vitro*. In addition, stimulation of granulosa cells by FSH may result in inappropriate differentiation of these cells during the early stages of folliculogenesis.

During follicular development, turnover and reconstruction of the basement membrane is facilitated and regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). When MMP-9 was secreted by preantral follicles *in vitro*, the probability of follicles having healthy granulosa or theca cells at the end of culture was 0.85 and 0.60, respectively. If TIMP-1 was released, there was a probability of 0.79 that the follicles would have healthy somatic cells. When TIMP-2 was detected, the probability of granulosa and theca cell health was 0.78 and 0.67, respectively. These results indicate that MMP-9 and TIMPs are related to follicular health, and can therefore be used as markers of follicular development.

Ascorbic acid has been implicated in several processes associated with follicular development, including collagen biosynthesis, steroidogenesis and apoptosis. The effect of this vitamin on the development of bovine preantral follicles was investigated during a twelve day culture period. Ascorbic acid had no effect on follicular growth or oestradiol secretion. Serum addition from Day 0 stimulated follicular growth but compromised follicular integrity. By Day 12 of culture, a higher proportion of follicles remained intact in the presence of ascorbic acid in serum-free conditions, with significantly less granulosa and theca cell death than control follicles. Moreover, ascorbic acid significantly increased production of MMP-9, an

enzyme involved in basement membrane remodelling and a marker for follicular health. Therefore, this culture system is capable of supporting follicle growth and differentiation over 12 days. Furthermore, ascorbic acid maintains bovine follicular health and remodelling *in vitro*.

Although IGF-1 did not increase follicular growth and oestradiol secretion *in vitro*, this factor did stimulate granulosa cell proliferation in preantral follicles during a six day culture. However, LR3 IGF-1, which does not bind to IGF binding proteins, was found to have a damaging effect on oocyte development, whereas recombinant IGF-1 promoted oocyte growth *in vitro*. These experiments highlight the importance of the IGF system in the early stages of follicular development, and emphasise the need for preserving physiological control mechanisms during follicle culture.

As well as adding to our understanding of the complex processes involved in follicular development, this research has led to the improvement of culture conditions for bovine preantral follicles and has identified markers of follicular health *in vitro*. A technique for obtaining a source of homogeneous mature oocytes from bovine ovaries would provide a model for *in vitro* maturation (IVM) and fertilisation (IVF) systems for human oocytes, as well as facilitating investigations into postovulatory and embryonic development.

Publications arising, or containing work from this thesis

Published papers

McCaffery FH, Leask R, Sršen V, Riley SC, Spears N, Telfer EE. Ascorbic acid maintains health and morphology of bovine preantral follicles during long term culture. Reproduction 2001; In Press

McCaffery FH, Leask R, Riley SC, Telfer EE. The culture of bovine preantral follicles in a serum-free system: markers for growth and development. Biol Reprod 2000; 63: 267-273

Telfer EE, Binnie JP, McCaffery FH, Campbell BK. *In vitro* development of oocytes from porcine and bovine primary follicles. Mol Cell Endocrinol 2000; 163: 117-123

Abstracts of oral presentations

McCaffery FH, Leask R, Riley SC, Telfer EE. The culture of bovine preantral follicles in a serum-free system: markers for growth and development. Theriogenology 2000; 53: 376

McCaffery FH, Telfer EE. The detection of matrix metalloproteinases and their inhibitors during the development of bovine preantral follicles *in vitro*. J Reprod Fertil Abstr Series 1999; 24: Abstract 15

McCaffery FH, Binnie JP, Leask R, Riley SC, Telfer EE. *In vitro* development of bovine follicles. Proceedings of the 4th National Ovarian Workshop 1999; Abstract 23

McCaffery FH, Telfer EE. The development of bovine oocytes *in vitro*. Proceedings of the 3rd National Ovarian Workshop 1998; Abstract 9

Abstracts of poster presentations

McCaffery FH, Telfer EE. The effects of ascorbic acid on the development of bovine preantral follicles *in vitro*. J Reprod Fertil Abstr Series 2000; 25: Abstract 224

McCaffery FH, Binnie JP, Leask R, Riley SC, Telfer EE. *In vitro* development of bovine preantral follicles. Abstracts of Eshre Campus Mammalian Follicle and Oocyte Culture 1999; 24-25

Acknowledgements

There are a number of people I wish to thank for their help and advice during the preparation of this thesis. Firstly, I would like to express my gratitude to my supervisor, Evelyn Telfer, for her endless enthusiasm and encouragement over the years. I could not have had a better role model. Thanks to everyone from the lab: Monica Bark, John Binnie and Kirsty Walters. Thanks especially to John for help with collection of ovaries, follicle dissection and histology. Colin MacDougall was also brilliant for brainstorming sessions and discussion of outlandish theories.

Many thanks to Simon Riley for allowing me to pursue my interest in MMPs and TIMPs, and for preparing the zymography figures for this thesis. Thank you for reading manuscripts, and always being on hand to offer advice. I also thank Rosemary Leask for sharing her expertise in gelatin zymography, and for performing some of the gelatin zymograms and the reverse zymograms in chapters 4 and 5.

Thanks to Bruce Campbell for allowing me to work in his laboratory and for performing the oestradiol assay in chapter 3. I am also grateful to Caroline Mutch, Alex Mitchell, and Carlos Souza for their assistance with the assays.

I would like to thank Norah Spears for allowing me to work in her laboratory and for offering advice on the ascorbic acid data. Alison Murray and Michael Molinek have also been a great help. Thanks also to Vlastimil Sršen for sharing his expert knowledge of TUNEL with me, and to Linda Sharp for assistance with confocal microscopy.

I would like to thank the Ministry of Agriculture, Fisheries and Food for supporting this work.

Finally, I thank David Thomas not only for his excellent statistical advice, but for being my best friend and a constant support to me throughout.

For Mum, Dad and David

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Abbreviations

ABC	avidin-biotin complex
AF	amniotic fluid
AFR	ascorbyl free radical
ANOVA	analysis of variance
bFGF	basic fibroblast-like growth factor
BMP	bone morphogenetic protein
BSA	bovine serum albumin
cAMP	3', 5'-cyclic adenosine monophosphate
DAB	3', 3'-diaminobenzidine tetrahydrochloride
DHAA	dehydro-L-ascorbic acid
DNA	deoxyribonucleic acid
dUTP	2'-deoxyuracil 5'-triphosphate
E_2	oestradiol 17β
ECM	extracellular matrix
EGF	epidermal growth factor
FBS	foetal bovine serum
FSH	follicle stimulating hormone
GDF-9	growth/differentiation factor 9
GnRH	gonadotrophin releasing hormone
GOC	granulosa cell-oocyte complex
HSPG	heparinsulphate proteoglycan
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IgG	immunoglobulin G
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
kDa	kilodalton
LH	luteinising hormone
MMP	matrix metalloproteinase
mRNA	messenger RNA

NGF	nerve growth factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PF	paraformaldehyde
PMSG	pregnant mare serum gonadotrophin
RNase	ribonuclease
RT	room temperature
SAPU	Scottish antibody production unit
SCF	stem cell factor
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TGF	transforming growth factor
TIMP	tissue inhibitor of matrix metalloproteinases
Tris	tris(hydroxymethyl)amine-methane
TUNEL	terminal deoxy nucleotidyl transferase-mediated dUTP-biotin nick end labelling
VEGF	vascular epithelial growth factor

Chapter 1

General Introduction

1.1. Follicular Development

1.1.1. Primordial Follicles

Mammalian ovaries develop as two swellings, termed genital ridges, along the ventral side of the mesenephros. Primordial germ cells migrate from the yolk sac to the genital ridge, and proliferate by mitosis. These germ cells are referred to as oogonia once they become enclosed into sex cords located in the cortex of the female gonad. This occurs from day 30 of gestation in the cow (reviews: Van den Hurk *et al.*, 1997, 2000).

In mammals, mitosis of germ cells is generally completed before birth. During the mitotic period, the number of germ cells in bovine ovaries reaches approximately 2 million; however, the vast majority (90-99%) of these degenerate by the time the calf is born, and less than 1% of the non-degenerated oocytes will proceed to ovulation (Erickson, 1966). On completion of mitosis, oogonia enter meiosis, and are referred to as primary oocytes. During the first meiotic prophase the primary oocytes become surrounded by clusters of flattened somatic cells, or pre-granulosa cells that secrete a basal lamina thus forming primordial follicles. At the diplotene stage of the first meiotic prophase, meiosis is arrested (review: Van den Hurk *et al.*, 1997).

1.1.2. Initiation of Follicular Growth

Once the pool of primordial follicles has been established, and in response to an as yet unknown signal, follicles are gradually and continuously recruited to grow. This initial growth is independent of the pituitary gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH) (Peters *et al.*, 1973). The avascular nature of the area in the ovary surrounding the primordial follicles indicates that locally produced growth factors are likely to regulate this process (van Wezel and Rodgers, 1996; Hirshfield, 1991).

1.1.3. Follicle Proliferation and Differentiation

During the follicular growth period, the oocyte grows and the granulosa cells proliferate to form a multilaminar structure called a preantral follicle (Figure 1.1). During preantral development, the oocyte enlarges and the zona pellucida forms around the oocyte. The granulosa cells change from a flattened to a cuboidal shape at this time, and are the site of rapid synthesis of matrix components, including basal laminae (Rodgers *et al.*, 1998). Connective tissue fibres are arranged parallel to the follicular basement membrane, and subsequently hormone-producing large epithelial cells and a capillary network constitute the thecal layer (review: Van den Hurk *et al.*, 1997). Thecal cells differentiate during the course of follicular growth and play an integrated role with the granulosa cells in producing oestrogen, as well as secreting androgens and a number of growth factors.

Once the follicle reaches a species-specific size, it forms a fluid filled space called an antrum (Figure 1.1). At this stage, follicles become increasingly dependent on gonadotrophins for further growth and development. The mature follicle is thus a developmental unit with three cell types and two functions; the production of hormones and the female gamete (Richards *et al.*, 1995).

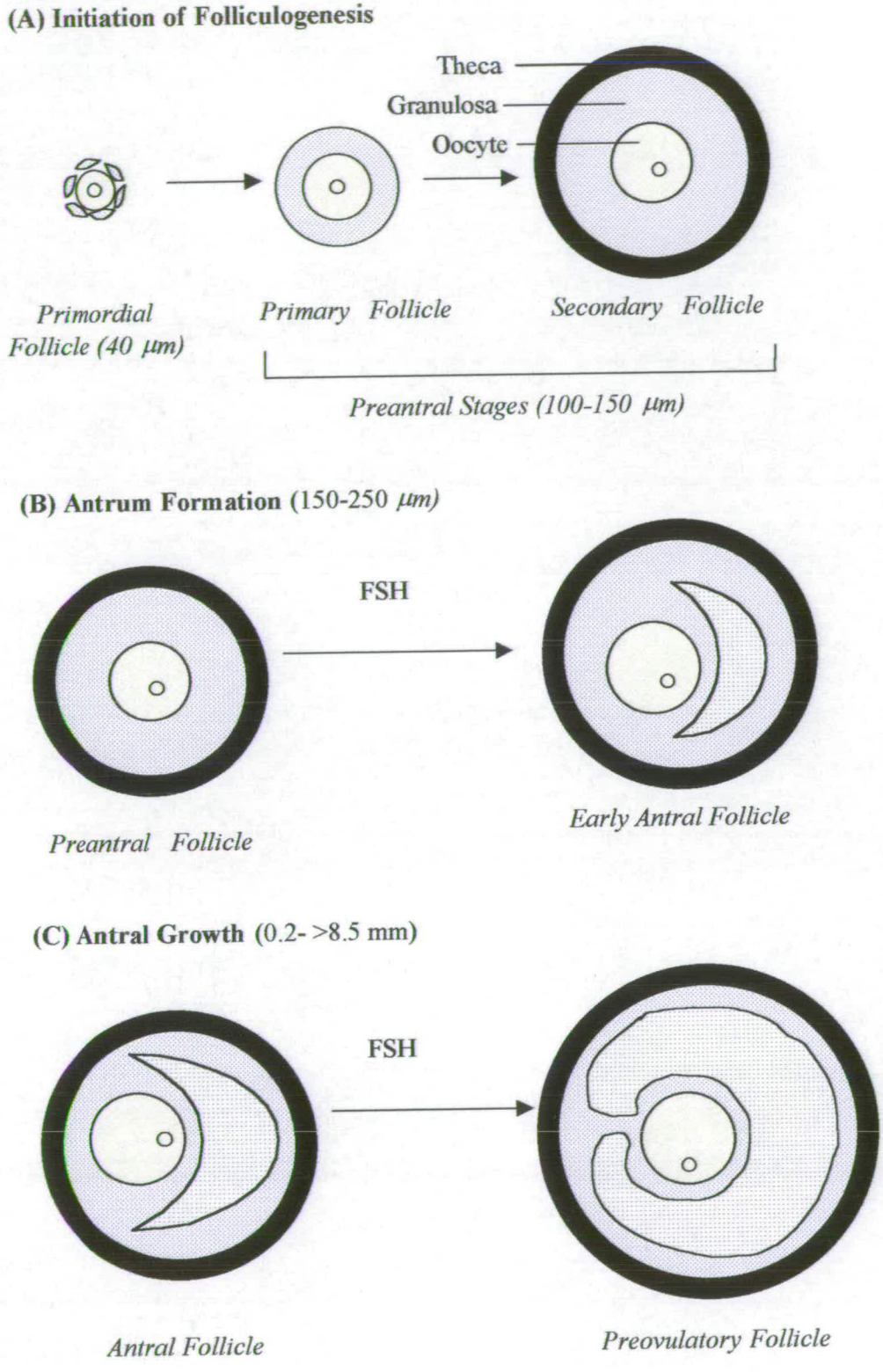


Figure 1.1. Follicular development. Follicle diameters (shown in brackets) from Lussier *et al.* (1994).

1.1.4. Oocyte Development and Maturation

Growing oocytes from immature follicles are not capable of resuming meiosis and are said to be meiotically incompetent. In rodents, it is generally accepted that oocytes stop growing and attain meiotic competence at approximately the time of antrum formation (Iwamatsu and Yanagimachi, 1975). However, in other species, the relationship between antrum formation, meiotic competence, and cessation of oocyte growth is not as well understood.

Using follicle size as an indicator of the competence of the oocytes they contain, oocytes from antral follicles larger than about 2 mm in diameter are thought to be fully grown and can resume meiosis spontaneously (cows and pigs: Motlik and Fulka, 1986). In these species, however, meiotic competence is acquired in two steps, based on the demonstration that pig and cow oocytes of 100 μm in diameter can resume meiosis but do not progress beyond Metaphase I, whereas cow oocytes of 110 μm and pig oocytes of 115 μm diameter complete meiotic maturation to Metaphase II (Fair *et al.*, 1995; Hyttel *et al.*, 1997; Motlik and Fulka, 1986). Hence oocyte size, rather than follicle size, is a more accurate indicator of meiotic competence.

It has been suggested that oocytes may have an autonomous chronological programme for acquisition of meiotic competence (Harada *et al.*, 1997). There may also be external stimulation by companion somatic cells in order for oocytes to become fully competent with a timing equivalent to that found *in vivo*. Harada and colleagues (1997) implicated the participation of FSH and hypoxanthine in the acquisistion of meiotic competence in growing bovine oocytes *in vitro*.

1.1.5. Follicular Oestrogen Synthesis

The ovary is the principal site of oestrogen production in the female (review: Zeleznik and Hillier, 1996). The principal oestrogen, oestradiol 17β (E_2), functions in the selection of follicles for ovulation during the reproductive cycle. The onset of follicular oestradiol secretion reflects a functional interplay between the two major steroidogenic cell types within the follicle (granulosa and theca) regulated by FSH and

LH. As illustrated in Figure 1.2, LH stimulates precursor androgen synthesis in the theca and FSH stimulates granulosa cell aromatase activity (Armstrong *et al.*, 1979). Thus both cell types and both gonadotrophins are thought to be necessary for oestrogen synthesis (Armstrong *et al.*, 1979; Hillier *et al.*, 1994)

1.1.5.1. Thecal androgen synthesis

Blood-borne cholesterol and acetate are converted into androgens within the vascularised theca interna, in which cytochrome P450 ($P450_{c17}$), the rate limiting enzyme in androgen synthesis (Sasano *et al.*, 1989), is abundantly expressed. This process is positively regulated by LH during antral development (Erickson *et al.*, 1985). IGF-1 and insulin receptors functionally coupled to androgen synthesis are also present on theca cells (Bergh *et al.*, 1991), and *in vitro* experiments have demonstrated that IGF-1 can enhance both basal and LH stimulated androgen production (Cara and Rosenfield, 1988; Hillier *et al.*, 1991).

1.1.5.2. Granulosa cell oestrogen synthesis

The avascular granulosa cells are exposed to androgens by diffusion from the theca interna. Once in the mural granulosa cell layer, androgens are converted into oestrogens through the action of aromatase (P450 arom), which is under FSH control (review: Zeleznik and Hillier, 1996). IGF-1 has also been shown to be capable of stimulating oestradiol and progesterone production in cultured human granulosa cells in a dose-dependent manner (Zhao *et al.*, 1998). In addition, both insulin and IGF-1 enhance basal progesterone and cAMP in cultured porcine granulosa cells (Sekar *et al.*, 2000), and stimulate oestradiol secretion and P450arom mRNA in cultured bovine granulosa cells (Manuel Silva and Price, 2000). The '2-cell, 2-gonadotrophin' mechanism for follicular oestradiol production is illustrated in Figure 1.2.

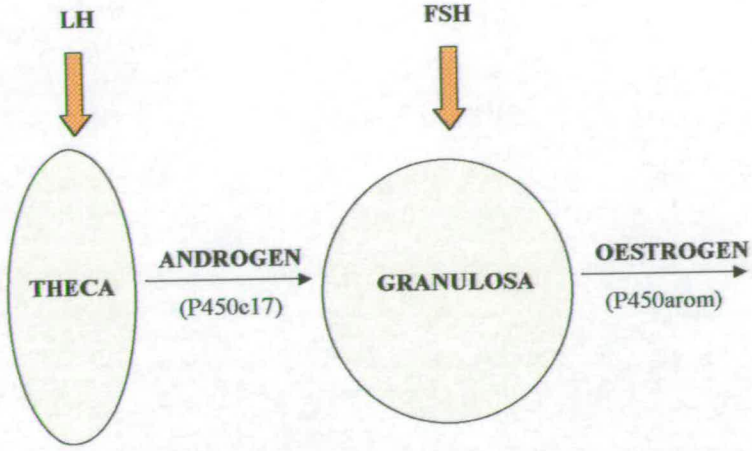


Figure 1.2. The '2-cell, 2-gonadotrophin' model of follicular oestrogen biosynthesis. Androgen synthesis, regulated by LH, occurs in the theca interna, catalysed by P450c17. Andogens are converted to oestrogens in the granulosa cells by P450arom in response to FSH. Adapted from Zeleznik and Hillier (1996).

1.1.6. Follicular Atresia

Less than 1% of follicles in the developing ovary will proceed to ovulation, whilst the rest will degenerate by a process called atresia (Erickson, 1966). This ensures that the correct number of oocytes reach maturity and are ovulated in each cycle. It is thought that atresia occurs through an apoptotic mechanism (Hughes and Gorospe, 1991; Tilly *et al.*, 1991), which, as originally defined by Kerr *et al.* (1972), is the best characterised form of physiological or programmed cell death.

Morphologically, apoptosis is recognised by detachment of a cell from its neighbours, cytoplasmic collapse and cell shrinkage (anoikis), condensation of chromatin and its redistribution to the nuclear perimeter (pyknosis), and the budding of spherical pieces of plasma membrane referred to as apoptotic bodies (Kerr *et al.*, 1972, 1994; Majno and Joris, 1995; Zakeri *et al.*, 1995; review: Martimbeau and Tilly, 1997). The main feature which distinguishes apoptotic cell death from necrotic cell death is the regulated action of Ca^{2+}/Mg^{2+} dependent endonucleases which cleave

the internucleosomal linker DNA of the nucleus into multiples of 185-200 base pairs (review: Martimbeau and Tilly, 1997). These DNA fragments can be detected using end labelling and separation by agarose gel elecrophoresis producing a characteristic laddering pattern (Wyllie, 1980). In addition, terminal deoxy nucleotidyl tranferase-mediated dUTP-biotin nick end labelling (TUNEL) utilises the activity of terminal deoxynucleotidyl transferase (TdT) enzyme to incorporate biotinylated dUTP onto the 3' ends of fragmented DNA (Gavrieli *et al.*, 1992).

Several factors have been identified which induce or suppress apoptosis (Tilly *et al.*, 1992; Billig *et al.*, 1993, 1994; Chun *et al.*, 1994). Anti-apoptotic factors include gonadotrophins, growth factors (e.g. EGF, TGFα, bFGF and IGF-1) and oestrogens. Factors which induce apoptosis include androgens and GnRH.

1.2. Investigation of Follicular Development

1.2.1. *In vivo* Studies

On the basis of gross and histological study of ovaries, it was proposed in 1960 that two waves of follicular activity occurred during the bovine oestrus cycles (Rajakoski, 1960). The wave pattern of follicular development refers to periodic, synchronous growth of a group of antral follicles. More recently, it has been shown that greater than 95% of bovine oestrus cycles are composed of either two or three follicular waves (Savio *et al.*, 1988; Sirois and Fortune, 1988; Ginther *et al.*, 1989; Adams *et al.*, 1994). Ultrasound tracking studies have proved useful in clarifying the number of waves per cycle as well as characterising the composition of follicular waves (Ginther *et al.*, 1989). However, this technique has not been successfully applied to detection of changes in preantral or early antral follicles during development.

Hormone treatment followed by ovariectomy, or techniques such as hypophysectomy have been useful for studying the function of gonadotrophins during early follicular development (e.g. Chiras and Greenwald, 1978; Nakano *et al.*, 1975). However, these experiments have limitations in terms of tracking follicular development over time.

In order to understand the complex mechanisms regulating folliculogenesis, it is useful to study natural or induced mutations that influence these processes. Transgenic mouse models have proved to be powerful tools for identification of key factors required for normal folliculogenesis (Matzuk, 2000). However, this technique cannot be readily applied to domestic ruminants. There is evidence of several major genes affecting prolificacy in sheep (Davis *et al.*, 2001), and these have provided a valuable model for the study of follicular growth and selection in large mammals. For example, the Booroola phenotype of increased litter size was originally selected in specific lines of Australian Merino sheep. Subsequent studies of sheep carrying the Booroola gene (FecB) confirmed segregation as a single autosomal locus (Davis *et al.*, 1982; Piper and Bindon, 1982). Ovarian follicles in Booroola Merinos, carrying the FecB gene, mature and ovulate at a smaller diameter than non-carriers of the

mutation (Webb *et al.*, 1999). Recently, Booroola sheep were shown to possess a mutation in the bone morphogenetic protein 1B (BMP 1B) receptor (ALK-6) that is expressed in both oocytes and granulosa cells (Shimasaki *et al.*, 1999). BMPs are members of the transforming growth factor-β (TGF-β) superfamily, which are multifunctional proteins that regulate growth and differentiation in many cell types. Another major gene (FecX) affecting ovulation rate has been found in Romney sheep (Davis *et al.*, 1991). The Inverdale fecundity gene (FecX), which maps to the sheep X chromosome, carries an inactivating mutation in growth/differentiation factor-9b (GDF-9b/BMP-15) (Galloway *et al.*, 2000), which is an oocyte specific factor (Dube *et al.*, 1998). Hence mutations in the BMP signalling system may contribute to the increased ovulation rate observed in Booroola and Inverdale animals. Interestingly, ewes that are homozygous for inactivation of GDF-9b/BMP-15 (Inverdale) are infertile due to a block in follicular growth, and those that are heterozygous for the mutation have increased ovulation rates (Galloway *et al.*, 2000). These findings establish that BMPs are important for female fertility and that natural mutations in an ovary-derived factor can cause both increased ovulation rate and infertility phenotypes in a dose-sensitive manner (Galloway *et al.*, 2000).

1.2.2. *In vitro* Studies

Normal folliculogenesis resulting in an oocyte capable of fertilisation and embryonic development depends upon a complex sequence of cellular interactions within the follicle. These create a unique and progressively shifting environment for the development of the oocyte. At any stage during this development, the follicle may continue normally or, more frequently, proceed towards atresia. To study the mechanisms involved, it is necessary to follow and influence the development of individual follicles *in vitro*.

Culture systems for preantral follicles, or their oocyte-granulosa cell complexes, are important for studying oocyte development, but are also vital for analysis of follicular somatic cell development and function. Growth of follicles in culture allows each follicle to be studied continuously and makes it possible to relate

particular developmental characteristics to subsequent reproductive performance of the gamete, thereby defining normal follicular development more accurately.

1.2.2.1. Rodent follicle culture systems

For successful oocyte growth *in vitro*, it is important to maintain the three-dimensional organisation of the granulosa cells and oocyte-somatic cell contact (Eppig, 1991). Therefore, culture systems developed using isolated granulosa cells (Gutierrez *et al.*, 1997b), whilst being valuable in determining the effects of growth factors and hormones, have limitations. Isolation techniques involving degradation of the theca cell layer and the basement membrane by collagenase results in granulosa cell-oocyte complexes (GOC), which have been cultured by Eppig and Schroeder (1989) to produce oocytes which have grown and have acquired developmental competence. As the oocyte itself is key to determining granulosa cell function, and the main role of the follicle is to support the development of the oocyte, culture systems for intact follicles or oocyte-granulosa cell complexes are preferable as they are more comparable to the environment *in vivo*.

Nayudu and Osborn (1992) used a manual dissection procedure to retrieve individual preantral follicles from the murine ovary. Because enzymatic digestion was not used, this system allowed the culture of preantral follicles with a theca layer and intact basement membrane. This was important for the examination of interfollicular effects and steroidogenesis. Murine follicle culture has also allowed the study of metabolism during folliculogenesis (Boland *et al.*, 1993).

The viability of the systems outlined above has been demonstrated by the production of live offspring from *in vitro* grown GOCs from murine preantral follicles (Eppig and Schroeder 1989) and from the culture of whole preantral follicles (Spears *et al.*, 1994). More recently, oocytes from primordial follicles activated *in vitro* have also acquired competence to be matured and fertilised, resulting in the production of a live mouse (Eppig and O'Brien, 1996).

1.2.2.2. Bovine follicle culture systems

As discussed above, techniques have been developed for isolating and culturing preantral follicles from a range of rodents and these have produced developmentally competent oocytes and viable offspring (Roy and Greenwald, 1989; Torrance *et al.*, 1989; Eppig and Schroeder, 1989; Spears *et al.*, 1994; Cortvrindt *et al.*, 1996). The limited success of these rodent systems has resulted in attempts to develop similar methods to be applied in humans (Abir *et al.*, 1997; Wright *et al.*, 1999) and domestic species (reviews: Telfer *et al.*, 2000; Van den Hurk *et al.*, 2000).

For livestock production, the culture of small oocytes could potentially provide a large population of female germ cells. Advances have been made in transferring aspects of the rodent systems to development of *in vitro* porcine follicle systems (Hirao *et al.*, 1994, Wu *et al.*, 2001). However, less densely packed follicles, more fibrous stromal tissue, larger follicles and slow follicular growth have all played a role in delaying a successful system for isolation and culture of bovine preantral follicles.

Complete *in vitro* growth of bovine preantral follicles is ambitious since preantral development is lengthy (4-5 months); therefore prolonged culture is necessary if developmentally competent oocytes are to be obtained. In addition, as the follicles increase in size, problems of nutrient restriction and insufficient gas and waste exchange may arise. In previous studies using bovine preantral follicles, culture was terminated long before preovulatory stage had been reached (Hulshof *et al.*, 1995; Ralph *et al.*, 1995b; Figueiredo *et al.*, 1995; Gutierrez *et al.*, 2000; Saha *et al.*, 2000). Nevertheless, preantral follicles from sheep and cattle have recently been grown to the antral stage of development *in vitro* (Cecconi *et al.*, 1999; Gutierrez *et al.*, 2000), indicating that follicle differentiation can be achieved under culture conditions. However, these follicle culture systems for domestic ruminants have been unsuccessful in obtaining oocytes capable of undergoing meiotic maturation.

1.3. Factors Affecting Follicular Development

There is little information on the regulation of primordial activation and subsequent preantral growth, with conflicting views on the role of specific factors in follicle growth and development.

1.3.1. Endocrine and Autocrine Regulation

Granulosa cells are the only cells in the female body to possess FSH receptors, and binding of FSH to the cell surface activates adenylyl cyclase and cyclic AMP-dependent protein kinase(s) leading to altered expression of multiple genes crucial to cytoproliferation and differentiation (review: Zeleznik and Hillier, 1996). Follicles can develop from the primordial to the late preantral stage in the complete absence of gonadotrophins (Dufour *et al.*, 1979). However, the involvement of FSH in the development of small follicles is supported by the presence of binding sites/receptors for FSH in granulosa cells of cows (Wandji *et al.*, 1992) and other mammals (Monniaux and de Reviers, 1989; Eckery *et al.*, 1997). Furthermore, FSH has been reported to activate granulosa cell proliferation and differentiation, and to reduce the number of atretic follicles grown *in vitro* (rodent: Tonetta and diZerega, 1989; Roy and Greenwald, 1989; Gore-Langton and Daniel, 1990; Nayudu and Osborn, 1992; Spears *et al.*, 1998; cow: Hulshof *et al.*, 1995; Wandji *et al.*, 1996; sheep: Newton *et al.*, 1999; human: Roy and Treacy, 1993; Abir *et al.*, 1997). The mitogenic action of FSH is facilitated by locally produced growth factors, production and/or action of which may be modified by FSH (Zeleznik and Hillier, 1996).

McNatty *et al.* (1979) reported that LH was required for oestrogen biosynthesis within the follicle (Figure 1.2). *In vitro*, murine follicles grown to the preovulatory stage have been shown to respond to LH by progesterone production (Qvist *et al.*, 1990) and ovulation (Boland *et al.*, 1993). Suitable balances between FSH and LH during a long term culture may be necessary in order to obtain optimum conditions for follicle and oocyte development.

Oestrogens are well known endocrine and intrafollicular autocrine mitogenic

compounds (Tonetta and diZerega, 1989). In cultured pig large preantral follicles, oestradiol synergises with FSH to enhance granulosa cell proliferation and antrum formation (Hirao *et al.*, 1994). In contrast, oestradiol has not been found to affect the proliferative activity of granulosa cells, but does appear to increase the size of bovine cultured primary and small secondary follicles (Hulshof *et al.*, 1995).

1.3.2. Intraovarian Regulation

1.3.2.1. Ovarian peptides

There is growing evidence that intraovarian peptides such as growth factors, extracellular matrix (ECM) compounds and neuropeptides exert paracrine communication within follicles. During preantral follicle development, growth factors such as epidermal growth factor (EGF); basic fibroblast-like growth factor (bFGF); transforming growth factors (TGFs); vascular epithelial growth factor (VEGF) and nerve growth factors (NGFs) may influence folliculogenesis (review: Van den Hurk *et al.*, 1997). As will be discussed later, the IGF system may also be a key regulator of early folliculogenesis.

The TGF-β superfamily is made up of a number of proteins with the potential to act as intraovarian regulators of ovarian function (Armstrong and Webb, 1997). For example, growth/differentiation factor-9 (GDF-9), a member of the TGF-β superfamily, is an oocyte-derived factor which has been reported to be essential for normal follicle development beyond the primary (one granulosa layer) follicle stage in mice (Dong *et al.*, 1996). TGF-βs may also play a significant role in folliculogenesis in ruminants. For example, as discussed earlier, mutations in the TBF-β signalling system have been implicated in the increased ovulation rate observed in Booroola and Inverdale sheep (Wilson *et al.*, 2001; Galloway *et al.*, 2000). In cattle, TGF-βs inhibit granulosa and theca cell proliferation while enhancing gonadotrophin-stimulated steroidogenesis (Roberts and Skinner, 1991), and bovine theca cells have been shown to produce TGF-β in culture (Lobb and Dorrington, 1992). In addition, it has been hypothesised that activin and inhibin, which are disulphide-linked dimeric

glycoproteins belonging to the TGF-β family, play an autocrine/paracrine role in controlling early follicular development by promoting follicular growth and differentiation (Findlay, 1993; Mather *et al.*, 1997). Intraovarian actions of granulosa cell-derived activins include the promotion of granulosa cell proliferation and upregulation of FSH receptors, P450arom, oestrogen synthesis, granulosa cell LH receptors and enhancement of oocyte maturation (review: Knight and Glister, 2001). Recently, it was reported that activin produced by secondary mouse follicles suppresses the growth of primary follicles *in vitro* (Mizunuma *et al.*, 1999), and it was hypothesised that a local decline in activin as a result of atresia of secondary follicles initiates early folliculogenesis in response to circulating FSH (Mizunuma *et al.*, 1999). In addition to an endocrine feedback role, granulosa-derived inhibins can sensitise theca cells to LH, thereby enhancing the production of androgens, an essential requirement for follicular oestrogen synthesis (Knight and Glister, 2001). The autocrine and paracrine actions of inhibin on enhancement of gonadotrophin stimulated steroid secretion by ovine and bovine granulosa and theca cells has recently been demonstrated (Webb *et al.*, 1999).

1.3.2.1.1. Follicular extracellular matrix

1.3.2.1.1.1. Composition and function

The extracellular matrix (ECM) provides a 'scaffolding' within a tissue to which cells attach, and contains domains that bind to specific cell surface receptors to modulate cellular activity. For example, ECM proteins interact with integrin receptors; transduce cytoplasmic signals; modulate the bioavailability of growth factors; and play a role in paracrine regulation of cellular differentiation, proliferation and apoptosis.

The membrana granulosa of each follicle is enveloped by a follicular basal lamina, or basement membrane, which separates it from the surrounding thecal and stromal cells (Gosden *et al.*, 1988; Luck, 1994; van Wezel and Rodgers, 1996). Basal laminas are composed of networks of collagen IV intertwined with laminin (Rodgers *et al.*, 1995). Fibronectin, heparinsulphate proteoglycans (HSPGs) and other molecules are associated with the collagen IV-laminin backbone. *In vivo*, extracellular matrix components such as laminin, fibronectin, collagens, and proteoglycans have been detected in follicles (Rodgers *et al.*, 1995). In bovine preantral follicles, collagen type IV and laminin have been detected in the basement membrane (which separates the granulosa and thecal layers), and fibronectin has been immunohistochemically visualised around preantral follicles and in granulosa cells (Figueiredo *et al.*, 1995). In ovine follicles, Huet and colleagues (1997) reported parallel changes in ECM components and steroidogenic enzymes in theca and granulosa cells during growth and atresia. For example, follicle growth was characterised by an increase in fibronectin and type I collagen in theca interna and granulosa cells respectively. Atresia was characterised by increased fibronectin, laminin, and type IV collagen in the wall of granulosa cells (Huet *et al.*, 1997).

Basal laminas in different parts of the body differ in the ratio of their components, thus the unique composition of each basal lamina contributes to its specific functional properties (Engvall, 1993). The surface area of the bovine follicle has been estimated to double nineteen times during folliculogenesis, and different isoforms of collagen IV and laminin are present within the follicular basement

membrane at different developmental stages (Rodgers *et al*, 1998). These observations suggest that the follicular basal lamina is continually remodelled during development (van Wezel and Rodgers, 1996).

1.3.2.1.1.2. Remodelling

The enzymes responsible for degrading the proteinaceous components of the ECM are the matrix metalloproteinases (MMPs). These are zinc and calcium-dependent enzymes that include collagenases, gelatinases, stromelysins and the membrane bound metalloproteinases (Nagase, 1997). MMP activity is highly regulated and subject to control at several levels (Kleiner and Stetler-Stevenson, 1993). Two important points of regulation include activation of latent enzymes and association with tissue inhibitors of metalloproteinases (TIMPs). To date, 17 members of the MMP family and 4 members of the TIMP family have been identified (Table 1.1 and 1.2).

Table 1.1. MMP family of ECM proteinases. Adapted from Smith *et al.* (1999).

Family	Name	Matrix substrates of functions
Collagenase	MMP-1	Collagens I, II, III, VII, X
	MMP-8	Collagens I, II, III
	MMP-13	Collagens I, II, III
	MMP-18	Collagen I
Gelatinases	MMP-2	Gelatins, collagens IV, V, VII, X, XI, fibronectin, laminin
	MMP-9	Gelatins, collagens IV, V, XIV, fibronectin
Stromelysins	MMP-3	Gelatins, fibronectin, laminin, collagens III, IV, IX, X, vitronectin: activates proMMP-1
	MMP-10	Fibronectin, collagen IV
	MMP-20	Amelogenin
Membrane-type MMPs	MMP-14	Collagens I, II, III, fibronectin, laminin, vitronectin: activates proMMP-2 and proMMP-13
	MMP-15	Gelatin, fibronectin, laminin: activates proMMP-2
	MMP-16	Activates proMMP-2
	MMP-17	Not known
Others	MMP-7	Fibronectin, laminin, gelatins, collagen IV
	MMP-11	Weak activity on fibronectin, laminin, collagen IV, gelatins
	MMP-12	Elastin
	MMP-19	Not known

Table 1.2. TIMP family. Adapted from McIntush and Smith (1998).

Inhibitor	Relative molecular weight	Extracellular location
TIMP-1	28 000	Soluble in ECM and body fluids
TIMP-2	21 000	Soluble in ECM and body fluids
TIMP-3	24 000	Bound to ECM
TIMP-4	22 000	Not known

Evidence is emerging that MMPs and TIMPs not only remodel the extracellular matrix but also influence many cellular functions. For example, TIMP-1 is a multifunctional molecule that stimulates proliferation of various cell types (Edwards *et al.*, 1996) and promotes steroiodogenesis (Boujrad *et al.*, 1995). In addition, as discussed previously, interactions between ECM components and cell surface molecules regulate cell behaviour. In a changing environment, the ability of cells to proliferate, survive or differentiate also changes. Therefore, MMPs can alter cell behaviour through their action on the ECM.

Follicular development requires significant proliferation of granulosa and thecal cells. The role of growth factors in control of cell proliferation has been studied extensively. Many growth factors are secreted constitutively and sequestered in the ECM in an inactive form or in association with specific binding proteins where they can be subsequently liberated by proteolysis of the ECM (Logan and Hill, 1992). Therefore, MMPs may play a key role in the regulation of the availability of growth factors and their activities within developing follicles.

1.3.2.1.2. Insulin-like growth factor system

Insulin has been shown to have a dose-dependent stimulatory effect on granulosa cell proliferation (Saumande, 1991; Langhout *et al.*, 1991; Peluso *et al.*, 1995). Similarly, insulin-like growth factors (IGFs), and particularly IGF-1, have been identified as important paracrine regulators of ovarian function. IGF-1 has been shown to be an absolute requirement for normal ovarian function, as mice in which IGF-1 gene expression was disrupted showed reduction in ovarian weight and failed to ovulate (Baker *et al.*, 1996).

Within bovine antral follicles, there is evidence of a complete intrafollicular IGF system (Armstrong *et al.*, 1998, 2000) in which follicles express IGF ligand, and mediation of IGF action and bioavailability occurs through receptors and binding proteins. As illustrated in Figure 1.3, the expression of mRNA encoding IGF-1 is developmentally regulated in a species-specific manner (Armstrong and Webb, 1997; Webb and Armstrong, 1998). Low levels of IGF-1 mRNA have been detected in theca cells of bovine antral follicles (Armstrong *et al.*, 2000), whereas in rodents and pigs, IGF-1 mRNA has been reported to be expressed by granulosa cells (Oliver *et al.*, 1989; Zhou *et al.*, 1996). In ruminants, various studies have demonstrated both the presence (Spicer *et al.*, 1993; Leeuwenberg *et al.*, 1995; Spicer and Echternkamp 1995; Yuan *et al.*, 1996) and absence (Perks *et al.*, 1995, 1999; Armstrong *et al.*, 1998, 2000) of IGF-1 mRNA in granulosa cells (Figure 1.3). Moreover, IGF-1 has not been found to be produced by ovine or bovine granulosa cells *in vitro* in the absence of luteinisation (Wathes *et al.*, 1995; Gutierrez *et al.*, 1997a; Armstrong *et al.*, 2000).

Investigations into the expression of IGFs within bovine follicles have indicated that IGF-11 is the predominant IGF *in vivo* (Armstrong *et al.*, 2000). However, type 1 IGF receptor expression has been demonstrated in follicles at an earlier stage than IGF-11 mRNA has been detected. Therefore, it has been hypothesised that preantral follicles may be regulated *in vivo* by IGFs from the circulation (Armstrong *et al.*, 2000). IGF-11 mRNA is expressed in theca cells of bovine follicles at around the time of antrum formation (Armstrong *et al.*, 2000)

(Figure 1.3), and the timing of expression of IGF-11 correlates with acquisition of LH receptors in the theca cells. Since IGF-1 has been shown to increase LH receptor number in bovine theca cells (Stewart *et al.*, 1995), it can be speculated that *in vivo*, IGF-11 takes the place of IGF-1 and regulates the timing of onset of LH receptor expression in theca tissue via an autocrine mechanism (Armstrong *et al.*, 2000).

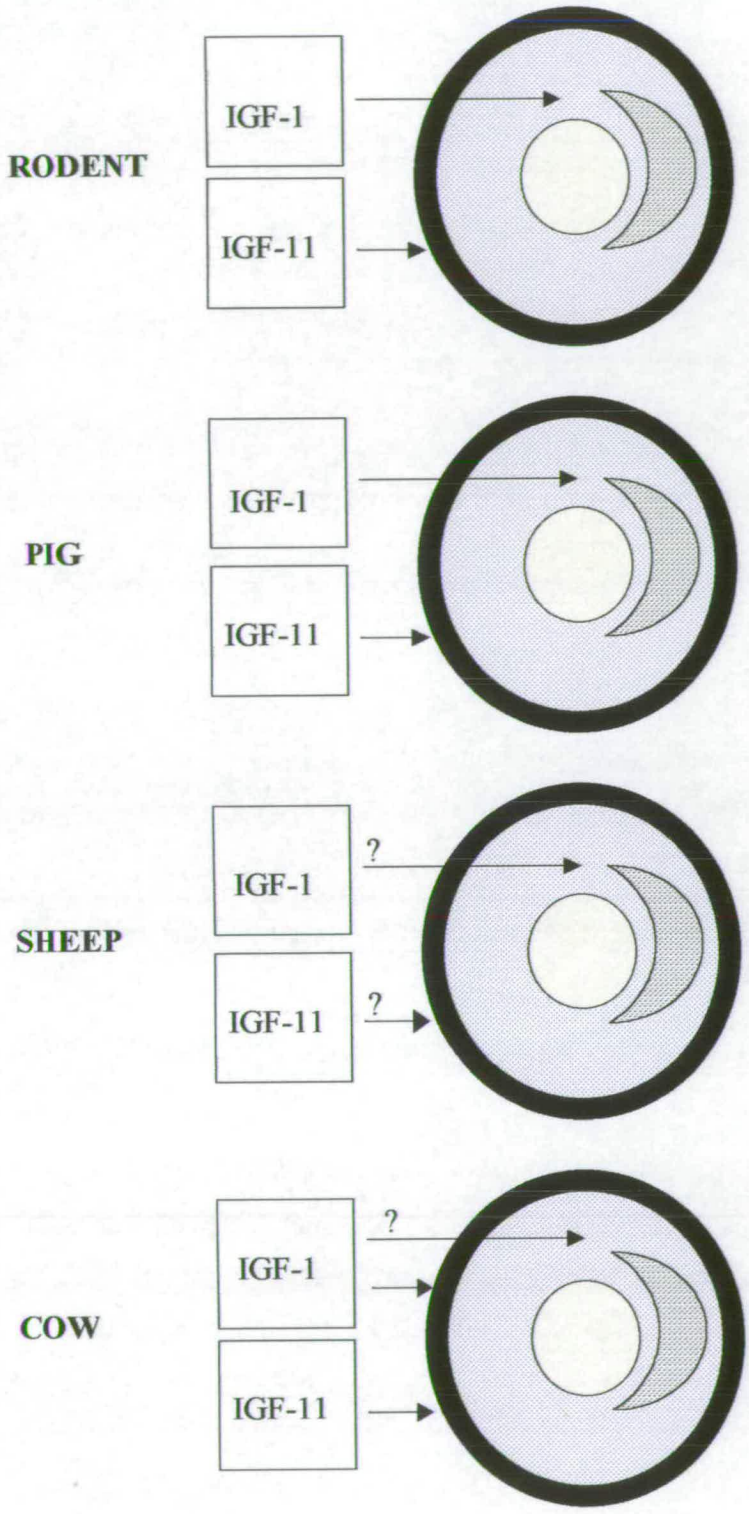


Figure 1.3. Follicular IGF expression

Previous studies have helped to determine the actions of IGFs in antral follicles, which are thought to vary according to species and the interaction of gonadotrophins (Giudice, 1992). For example, granulosa cells from bovine antral follicles show increased proliferation and oestradiol production in response to IGF-1 (Gutierrez *et al.*, 1997b). IGF-1 has also been shown to stimulate proliferation and oestradiol secretion in the absence of FSH (Adashi and Roban, 1992). Although the precise role of the IGF system at earlier stages of follicle development is poorly understood, IGF-1 binding has been demonstrated in bovine preantral follicles (Wandji *et al.*, 1992) and type 1 IGF receptor mRNA has been detected in bovine oocytes, granulosa and theca cells from the preantral stage (Armstrong *et al.*, 2000).

The bioactivity of IGFs is controlled by their association with a family of specific IGF binding proteins (IGFBPs), which are found in association with the extracellular matrix (ECM) and cell membranes. To date, expression of mRNAs encoding IGFBP-2 to –5 have been found in bovine follicles and expression of IGFBP-2, -4 and –5 in ovine follicles (Armstrong and Webb, 1997; Webb and Armstrong, 1998). IGFBP-2 and –4 mRNA expression in non-atretic bovine follicles is confined to granulosa and theca tissue, respectively (Armstrong *et al.*, 1998). During follicular development and atresia, there are distinct changes in the temporal and spatial expression of these proteins *in vivo*. For example, during the development of follicular dominance, intrafollicular amounts of IGFBP-2, -4 and –5 are decreased, whereas during atresia, the levels of these proteins increase (Austin *et al.*, 2001). Thus changes in the expression of IGFBPs, coupled with changes in the activity of IGFBP-specific proteases, provide a mechanism to regulate the bioavailability of IGFs during folliculogenesis (Armstrong *et al.*, 1998). Since IGFBPs have equal or higher affinity for IGFs than do IGF receptors (Shimasaki *et al.*, 1991), the degradation of IGFBPs by specific IGFBP-degrading proteinases may be an important step in regulating the actions of IGFs (Figure 1.4).

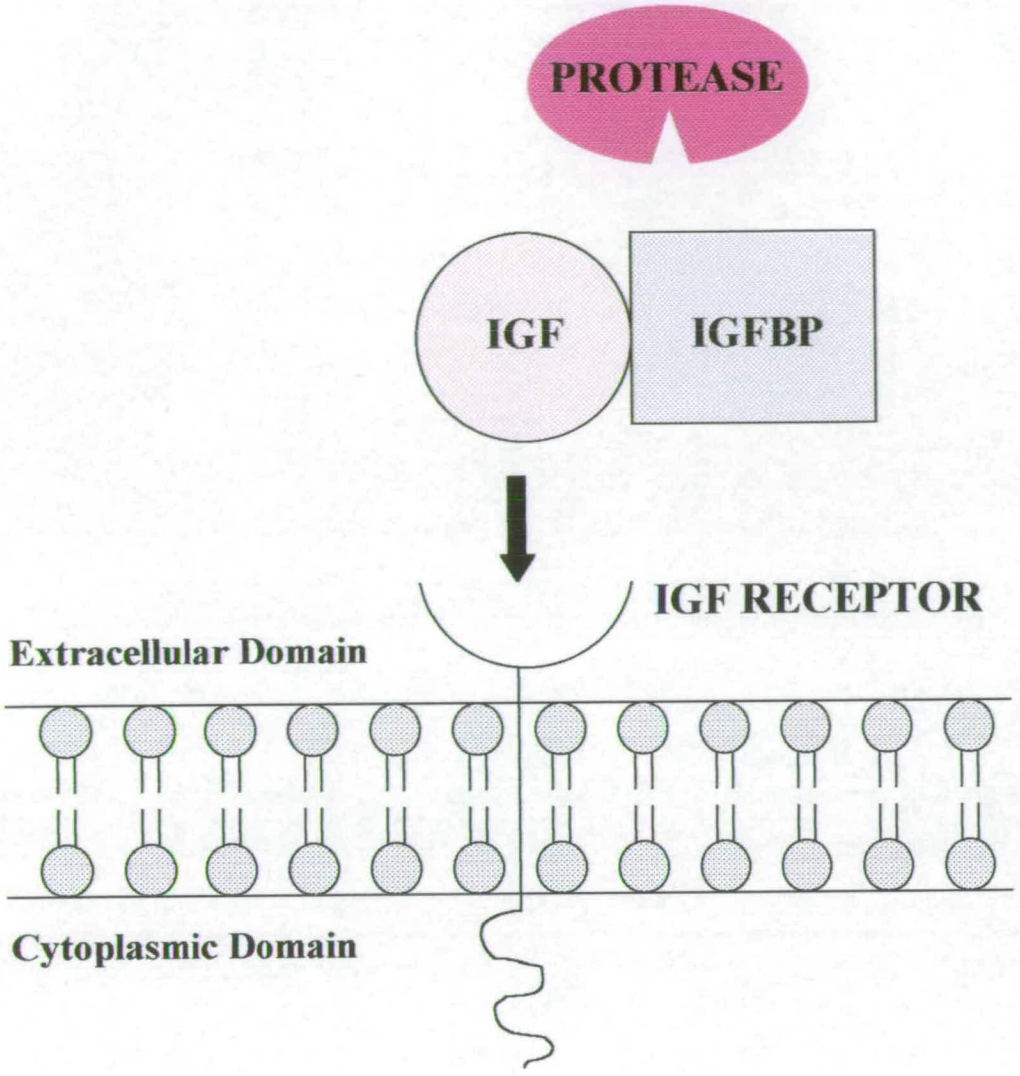


Figure 1.4. Mechanism of IGF action. IGFs are stored in extracellular depots in association with binding molecules (IGFBPs). IGFs can be liberated and functionally activated by the action of IGFBP-specific proteases which partially degrade IGFBPs resulting in a reduced affinity for the ligand versus that of the tissue receptors, or by alteration of binding protein affinity through its phosphorylation (Logan and Hill, 1992).

1.3.2.2. Oocyte-specific factors

The three dimensional organisation of the cells and the interactions of the various follicular cell types is critical for normal cell development and function. It is becoming increasingly apparent that the oocyte plays a crucial role in somatic cell development and function, as well as *vice versa* (Buccione *et al.*, 1990; Vanderhyden *et al.*, 1990, 1992). Studies regarding the molecular aspects of follicle and oocyte development are still very much at a primary stage. Preliminary characterisation of the cumulus expansion enabling factor in mouse oocytes (Eppig *et al.*, 1993) has been instrumental in gaining knowledge of the interactions between the oocyte and surrounding somatic cells, but the mechanisms governing oocyte-specific gene expression are still unknown. The enabling factor has been detected in bovine oocytes, but is not essential for cumulus expansion (Ralph *et al.*, 1995a), which suggests either a different function or a different mode of action for the secreted factor in this species.

Growth/differentiation factor-9 (GDF-9), a member of the TGF-β superfamily, is an oocyte-derived factor which has been reported to be essential for normal follicle development beyond the primary (one granulosa layer) follicle stage in mice (Dong *et al.*, 1996). This novel growth factor has been shown to be expressed in human and mouse ovaries, and appears to be localised exclusively to oocytes at all stages of follicular growth except primordial follicles in neonatal and adult mice (McGrath *et al.*, 1995). The pattern of GDF-9 expression and results from GDF-9 gene knockout studies in mice suggest that this factor may play an autocrine role in the regulation of oocyte development and maturation and/or a paracrine role in the regulation of granulosa cell proliferation and differentiation. Mouse studies (Dong *et al.*, 1996; Elvin *et al.*, 1999) have also shown that in the absence of GDF-9, follicles are incompetent to emit a signal that recruits theca cell precursors to surround the basement membrane (Elvin *et al.*, 1999). Recently, the expression of GDF-9 mRNA has also been demonstrated in bovine and ovine oocytes from the primordial follicle stage (Bodensteiner *et al.*, 1999). An oocyte-specific homologue of GDF-9, named BMP-15 (GDF-9b) has also been cloned in mice (Dube *et al.*, 1998). As discussed previously, the Inverdale fecundity gene (FecX) carries an inactivating mutation in

BMP-15 (Galloway *et al.*, 2000), implicating this factor in the control of ovulation rate in sheep.

The tyrosine kinase receptor c-kit and its ligand, stem cell factor (SCF), have been localised to oocytes and granulosa cells, respectively (Motro and Bernstein, 1993). Inhibition of the interaction between SCF and c-kit prevents the transformation of murine primordial follicles to primary follicles (Huang *et al.*, 1993; Yoshida *et al.*, 1997). In the sheep ovary, c-kit and SCF have been detected at all stages of follicular growth from the primordial phase (Clark *et al.*, 1996; Tisdall *et al.*, 1997). Thus the activation of the c-kit tyrosine kinase system by SCF appears to be an important process during early folliculogenesis.

1.4. The Role of Ascorbic Acid in Follicular Development

Ascorbic acid, or vitamin C, is synthesised from glucose in the liver of most mammals. However, primates, fruit eating bats and guinea pigs require a dietary intake of ascorbic acid, as they lack L-gulono-lactone oxidase, one of the enzymes necessary for its synthesis (Grollman and Lehninger, 1957). The critical functions of ascorbic acid in biological systems derive from its ability to donate electrons while itself undergoing reversible oxidation (Bielski *et al.*, 1975). This principle is illustrated in Figure 1.5.

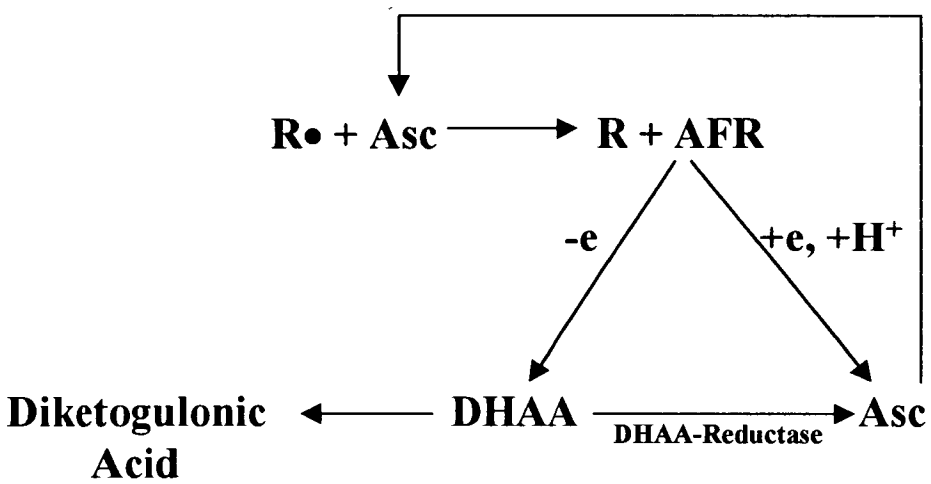


Figure 1.5. Mechanism of ascorbic acid action. A possible scheme by which a free radical species (R•) is neutralised by single electron transfer with ascorbic acid (Asc). This results in a detoxified product (R) and the ascorbyl free radical (AFR). Pairs of AFR disproportionate to form one molecule of dehydro-L-ascorbic acid (DHAA) and one Asc. DHAA can undergo a biologically irreversible opening of the lactone ring to form the inert product, diketo-L-gulonic acid; alternatively, it can be reduced to the useful Asc. From Rose and Bode (1993).

There are three biological actions of ascorbic acid which are relevant to reproduction. It is required for the biosynthesis of collagen, for the biosynthesis of steroid and peptide hormones and to prevent or reduce the oxidation of biomolecules (review: Luck *et al.*, 1995).

An example of the effect of ascorbic acid on connective tissue biosynthesis was presented by Jeffrey and Martin (1966), who showed a marked increase in the *in vitro* growth of embryonic chick bones in the presence of ascorbic acid, compared to bones grown in media without ascorbic acid. Acting as an electron donor, ascorbic acid is an essential co-factor for the enzymes that hydroxylate proline and lysine residues during the post-translational processing of pro-collagen (Pinnell, 1985). As these enzymes are necessary for collagen helix formation and cross-linking, in the absence of ascorbic acid, collagen would be structurally unstable and would not be properly secreted by the cell (Pinnell, 1985). In addition to its role as an enymatic co-factor, ascorbic acid has also been reported to stimulate collagen-specific mRNA in fibroblasts (Pinnell, 1985).

The ovarian content of ascorbic acid changes throughout the oestrus cycle. Prior to ovulation, ascorbic acid levels decline followed by an immediate rise in concentration, which facilitates progesterone production, consistent with the role of ascorbic acid in steroidogenesis (Byrd *et al.*, 1993). It has also been suggested that ascorbic acid at high concentrations inhibits steroidogenesis (Sanyal and Datta, 1979). The effects of this factor on hormone production during the early stages of folliculogenesis have not been elucidated.

The role of ascorbic acid as a free radical scavenger is well understood. Using cultured follicles, Tilly and Tilly (1995) reported that inhibitors of oxidative stress such as superoxide dismutase, ascorbic acid and catalase, can mimic the ability of FSH to prevent apoptosis *in vitro*. Moreover, recent investigations have shown that FSH and IGF-1, both of which are anti-apoptotic in granulosa cells (Chun *et al.*, 1994; Tilly and Tilly, 1995), increase ascorbic acid uptake by granulosa cells through activation of a membrane-associated ascorbate pump (Behrman *et al.*, 1996). Since follicles acquire responsiveness to gonadotrophins and grow rapidly during the late

preantral stages of follicle development, the ability to accumulate ascorbic acid may promote follicle survival.

1.5. Aims of Research

The aims of this thesis are:

(I) To investigate factors affecting early bovine follicular development using a defined culture system.

(II) To identify markers of early follicular development *in vitro*.

A limiting factor in reproductive methods such as IVF, embryo transfer, transgenesis and cloning is the lack of available fertilisable oocytes. Techniques such as superovulation can increase the number of oocytes ovulated by an individual, but this technology has limitations, as oocytes cannot be harvested from the large population of hormone-insensitive preantral follicles. Methods for *in vitro* maturation (IVM) of oocytes from antral follicles have been developed. However, the success rate and quality of embryos produced by IVM is low (reviews: Telfer *et al.*, 2000; Van den Hurk *et al.*, 2000). This may be explained by Telfer *et al* (1998a), who showed that 90% of antral follicles were undergoing apoptotic cell death at the time of isolation. In contrast, only 10% of preantral and early antral follicles showed any sign of apoptosis (Telfer *et al.*, 1998a). Therefore, development of a culture system which is capable of supporting preantral (and eventually primordial) follicles to a stage where the oocytes can be matured and fertilised *in vitro* may help to provide large amounts of homogeneous oocytes. Such a system may also allow identification of factors necessary for normal follicular development and acquisition of oocyte developmental competence.

In the preliminary experiments described in chapter 3, the effects of FSH and IGF-1 on follicular growth using a serum-free culture system will be determined. In addition, follicular differentiation will be assessed by measurement of oestradiol secretion throughout the culture period. In chapters 4 and 5, regulation of ECM remodelling *in vitro* will be investigated. The specific aims of chapter 4 are (1) to determine whether MMPs are secreted by bovine follicles *in vivo*; (2) to identify secretion of MMPs and TIMPs by preantral follicles *in vitro*, and assess these factors

as markers of follicular development; and (3) to determine whether IGF-1 affects MMP and TIMP secretion *in vitro*. Chapter 5 will assess the effects of ascorbic acid, a factor involved in basement membrane remodelling, on bovine follicle development *in vitro*. Follicle growth and basement membrane integrity will be assessed during the culture period, and the effect of ascorbic acid on follicular morphology, MMP production, oestradiol secretion and follicular cell death will be investigated. In chapter 6, we will further investigate the effects of IGF-1 on preantral follicle development *in vitro*, using histological and immunocytochemical methods. In addition, the effect of sequestration of IGF-1 by IGF binding proteins will be determined.

Chapter 2

General Materials and Methods

2.1. Follicle Isolation

2.1.1. Dissection Medium

Dissection medium was used for bench manipulation of ovarian material, and was prepared as follows. Leibovitz's medium (GIBCO BRL, Life Technologies Ltd, Paisley, Renfrewshire, UK) was supplemented with sodium pyruvate (2 mM), glutamine (2 mM), BSA (3 mg/ml), penicillin G (75 μg/ml) and streptomycin (50 μg/ml). Dissection medium was heated to 37 °C before use. All chemicals were from Sigma Chemicals, Poole, Dorset, UK unless otherwise stated.

2.1.2. Collection of Cortical Slices

Bovine ovaries from random stages of the oestrous cycle were obtained from an abattoir and transported at 25-30 °C in M199 (HEPES buffered) media supplemented with sodium pyruvate (2 mM), glutamine (2 mM), BSA (3 mg/ml), penicillin G (75 μg/ml) and streptomycin (50 μg/ml). In a laminar flow hood, ovaries were rinsed with 70% ethanol, and fine slices of ovarian cortex were taken using a scalpel and placed in dissection medium.

2.1.3. Micro-dissection

In a petri dish under a dissecting microscope (Olympus, UK) fitted with a calibrated eyepiece graticule (Graticules Ltd, Tonbridge, Kent, UK), preantral follicles (100-200 μm) were isolated from the cortical slices using fine 25 G needles (Merck Ltd, Lutterworth, UK) attached to syringe barrels (Figure 2.1). Approximately 15 to 30 follicles were isolated on each dissection day. Follicles with an intact basement membrane and even distribution of granulosa and theca layers were selected for culture.

2.2. Follicle Culture

2.2.1. Culture Medium

Basic culture medium was prepared as follows. McCoy's 5a medium with bicarbonate and HEPES (20 mM) was supplemented with BSA (0.1%), L-glutamine (3 mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), transferrin (2.5 μg/ml), selenium (4 ng/ml), androstenedione (10^{-7}M) and insulin (10 ng/ml). Medium was filter sterilised (syringes: Merck; filters: Corning Costar, UK) and heated to 37 °C. This basic medium was used at the beginning of this research project. Ascorbic acid, as described below, was added to culture medium in later experiments.

2.2.1.1. Ascorbic Acid

During the culture of murine preantral follicles, 50 μg/ml ascorbic acid (L-ascorbic acid, sodium salt) was shown to improve follicle survival by reducing the incidence of basement membrane rupture, and by reducing apoptotic cell death (Murray *et al.*, 2001). Following this discovery, the effect of ascorbic acid was investigated in our culture system, as described in chapter 5. Subsequently, ascorbic acid was added to all culture medium. Aliquots of stock medium containing ascorbic acid were stored at –70 °C, and added fresh during culture medium changes.

2.2.2. Culture

Preantral follicles (100-200 μm) were cultured individually in 96 well plates (Bibby Sterilin Ltd, Stone, Staffs, UK) in 250 μl of culture medium in the absence of serum (Figure 2.1). Plates were incubated for 6 days in a sterile humidified air atmosphere with 5% CO_2 at 37 °C. Follicle diameters, taken on perpendicular planes, were measured under the dissection microscope on days 0, 2, 4 and 6. Half of the medium was replaced every second day, and this conditioned medium was stored at -20 °C until required. For each experiment, each set of cultures took place under identical conditions.

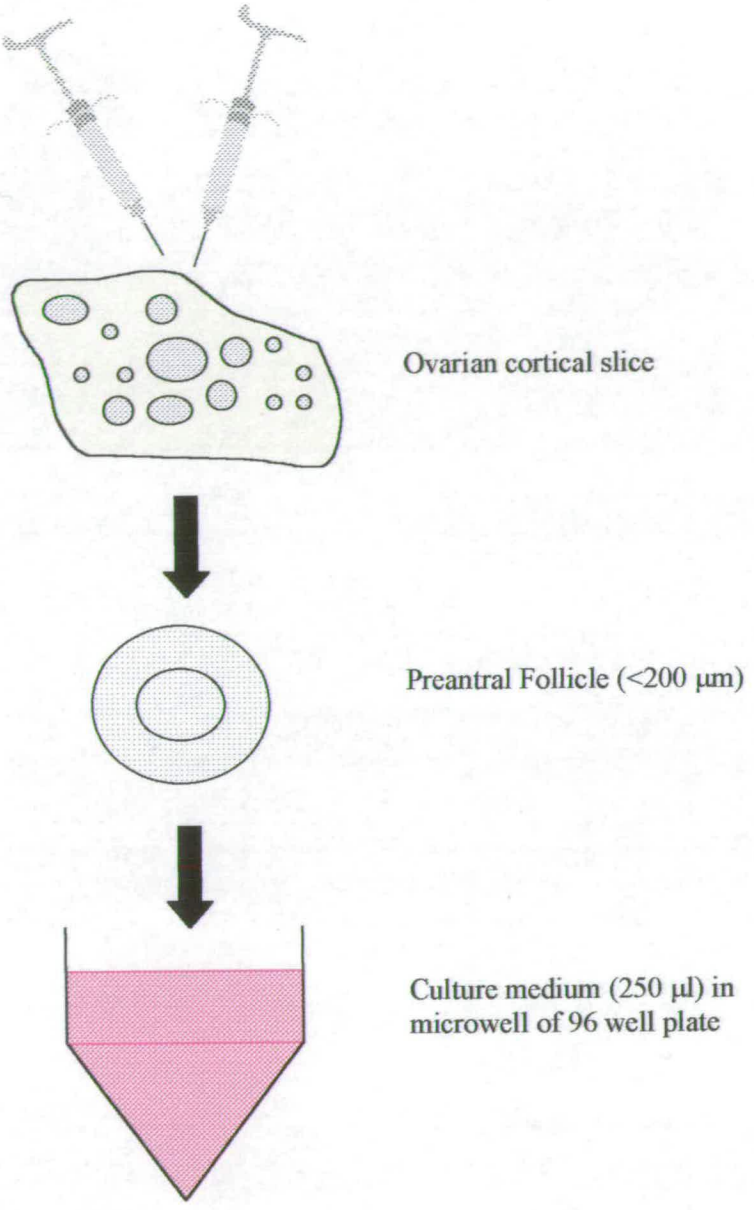


Figure 2.1. Isolation and culture of preantral follicles

2.3. Histology

2.3.1. Fixation

At the end of the culture period, follicles were fixed overnight in Bouin's solution (70% picric acid, 25% formaldehyde, 5% glacial acetic acid) or in 4% paraformaldehyde (4% paraformaldehyde in dH_2O, 0.1M NaOH, 0.1M Na phosphate pH 7, 4 °C). The fixative was removed and replaced with 70% ethanol until required. Addition of a small amount of eosin (Merck) to the 70% ethanol aided visualisation of follicles during processing.

2.3.2. Processing of Follicles for Morphological Assessment

2.3.2.1. Processing of samples

Follicles for morphological assessment were dehydrated by repeated changes through a series of increasing ethanol concentrations up to absolute ethanol. Absolute ethanol was replaced with cedar wood oil (Merck) for a minimum of 24 hours, then the oil was cleared from the follicles using toluene (in a fume hood) for 30 min. Follicles were embedded in paraffin wax (Merck) at 60 °C, with changes every hour for 4 h to remove all traces of toluene.

2.3.2.2. Sectioning and mounting

The samples were sectioned at 6 μm using a microtome (Leica UK Ltd, Milton Keynes, UK). The sections were floated onto gelatin-coated slides (Merck) (prepared by dipping in a solution of 1% gelatine, 0.1% chromic potassium sulphate in dH_2O) in a water bath at 42 °C and allowed to dry overnight at 37 °C.

2.3.2.3. Staining

Sections were dewaxed in xylene for 15 minutes and taken down through a series of ethanol concentrations (absolute to 70%). The staining procedure was as follows (for reagent preparation see Drury and Wallington, 1976): Bouins fixative was removed from sections by dipping slides in 70% ethanol with lithium carbonate. Slides were rinsed in tap water and placed in Harris' Haemotoxylin (5 min), rinsed in water again and placed in Scott's tap water substitute. Sections were rinsed in tap water and placed in Eosin (2 min, 1:1 solution), dipped in tap water and placed in potassium alum (Merck) (3 min), and rinsed again. The sections were dehydrated through a series of ethanol concentrations and placed in xylene. The sections were mounted using DPX mounting medium (Merck) and a glass coverslip.

2.3.3. Processing of Follicles for Immunocytochemistry

2.3.3.1. Processing of samples

Follicles were processed and embedded as described in 2.3.2.1.

2.3.3.2. Sectioning and mounting

The samples were sectioned at 6 μm using a microtome (Leica), mounted on charged slides (Merck), and allowed to dry overnight at 37 °C. Slides were stored until required.

2.3.3.3. Immunocytochemistry

Samples were dewaxed in xylene and rehydrated from ethanol to distilled water, and washed in PBS (2x5 min, Sigma). Antigen retrieval was performed by placing sections in 0.01M citrate buffer (10 min microwave (800W), 20 min at RT, 0.19% citric acid (anhydrous) in dH_2O, pH 6). Sections were washed in PBS (2x5 min), placed in 3% hydrogen peroxide for 10 min and washed (2x5 min, PBS). Sections

were placed in horse serum from a Vectastain ABC-Elite kit (Vector Laboratories, Southgate, UK) for 20 min at RT, followed by incubation at 37 °C with primary antibody (1 hr, 85 μg/ml Ki67-MM1 [Novocastra Laboratories, Newcastle Upon Tyne, UK]). Negative controls were incubated with 1 mg/ml mouse IgG (Novocastra Labs) for 1 hr at 37 °C. Sections were washed (2x5 min, 1% Tween 20 in PBS) and incubated with a biotinylated secondary antibody (Vector Labs, as kit) for 30 min at RT, and washed (2x5 min, 1% Tween 20 in PBS). Slides were incubated with a tertiary antibody (Vector Labs, as kit) for 30 min at RT, and washed (2x5 min, 1% Tween 20 in PBS). Localisation of the antigen was performed by incubation with DAB (Vector Labs, as kit) for 6 min. Slides were washed in dH_2O, counterstained with haematoxylin, dehydrated and mounted.

2.3.4. Collection of Histological Results

Histological measurements and observations were made under the light microscope with a crossed micrometer (Graticules Ltd). The section containing the oocyte nucleolus, or if this was absent, the largest cross-section of the oocyte was used for observations and measurements. Follicle and oocyte sizes were measured, and proliferation was assessed by counting the number of granulosa and theca cell layers. Granulosa cell death was measured by counting the number of pyknotic cells and expressing them as a percentage of the total number of granulosa cells: atretic follicles were defined as those with >5% pyknotic nuclei (Byskov, 1974). Theca cell health was assessed in two ways: (a) by the level of pyknosis, as defined above, and (b) by the number of layers present, with < 3 layers indicating theca degeneration. Oocytes were classed as healthy only if they were morphologically normal, and contained an intact germinal vesicle. Misshapen oocytes, or those that were absent or contained no germinal vesicle were classed as degenerate.

Chapter 3

Growth and Differentiation of Bovine Preantral Follicles in a Serum-free Culture system

3.1. INTRODUCTION

Ovarian folliculogenesis is regulated by both endocrine and intraovarian mechanisms which co-ordinate the processes of somatic cell proliferation and differentiation. As discussed in chapter 1, FSH and IGFs are thought to be principal regulators of follicular development.

Follicles can develop from the primordial to the late preantral stage in the complete absence of gonadotrophins (Dufour *et al.*, 1979). However, the involvement of FSH in the development of small follicles is supported by the presence of binding sites/receptors for FSH in granulosa cells of cows (Wandji *et al.*, 1992) and other mammals (Monniaux and de Reviers, 1989; Eckery *et al.*, 1997). Furthermore, FSH has been reported to stimulate growth (Qvist *et al.*, 1990; Gutierrez *et al.*, 2000); antrum formation (Roy and Greenwald., 1989; Hirao *et al.*, 1994; Gutierrez *et al.*, 2000); oestradiol production (Qvist *et al.*, 1990) and LH responsiveness (Boland *et al.*, 1993) of preantral follicles *in vitro*. As the influence of FSH varies during follicular growth and development (Nayudu and Osborn, 1992; Qvist *et al.*, 1990), account must be taken of this in a long term culture system.

IGFs influence a variety of biosynthetic processes in ovarian granulosa and theca cells, as well as mitotic activity in ovarian granulosa cells (Giudice, 1992). This intraovarian IGF autocrine/paracrine system appears to regulate ovarian follicular development and steroidogenesis, either alone or in synergy with gonadotrophins (Giudice, 1992). The IGF system has been well characterised in antral follicles, but there are fewer studies on the effects of IGFs at earlier stages of follicular development. In mice, IGF-1 is associated with the growth and survival of rapidly expanding large preantral and early antral follicles (Adashi *et al.*, 1997; Wandji *et al.*, 1998). However, differences in temporal and spatial production of IGF-1 between rodents and domestic species suggests different mechanisms of action (Leeuwenberg *et al.*, 1995; Yuan *et al.*, 1996; Gutierrez *et al.*, 1996; Wandji *et al.*, 1998; Schams *et al.*, 1999).

The aims of our initial experiments were to determine the action of FSH and IGF-1 on the growth of bovine preantral follicles in a serum-free culture system. This work was initiated after Ralph (1996) implicated a role for FSH in stimulation of

bovine preantral follicle development *in vitro*. Subsequently, Gutierrez *et al.* (2000) used a long-term serum-free culture system to determine that both FSH and IGF-1 were beneficial to bovine preantral follicle and oocyte development. Ideally, it is preferable to use a serum-free system in order to assess the requirements for normal follicular development without the presence of unknown serum factors.

In the current chapter, preliminary experiments to investigate the effects of FSH and IGF-1 on follicular growth using a serum-free culture system (Gutierrez *et al.*, 2000) were performed. In addition, follicular differentiation was assessed by measurement of oestradiol secretion throughout the culture period. One modification made to the basic culture system was the inclusion of ascorbic acid in the culture medium in some of the experiments. In mouse preantral follicle culture, ascorbic acid was shown to improve follicle survival by reducing the incidence of basement membrane rupture, and by reducing apoptotic cell death (Murray *et al.*, 2001). Following this discovery, the effect of ascorbic acid on bovine follicular development *in vitro* was investigated. This will be discussed in depth in chapter 5. For the purposes of the current chapter, it should be noted that initial experiments were performed in the absence of ascorbic acid.

3.2. MATERIALS AND METHODS

3.2.1. Follicle Isolation and Culture

Preantral follicles (100-200 μm) were isolated from bovine cortical slices and cultured individually under the same conditions for 6 days as described in chapter 2.

3.2.2. Treatments

3.2.2.1. Treatment with LR3 IGF-1 and FSH in the absence of ascorbic acid

Basic culture medium (control) was prepared as described in 2.2.1. The analogue Long R3 IGF-1 (LR3 IGF-1), which does not bind to IGF binding proteins, was purchased from Gropep Pty Ltd, Adelaide, SA. The FSH (NIDDK-oFSH-20) was obtained from Dr Parlow, National Hormone and Pituitary program, Torrance, USA. The treatment groups were:

(a) (1) Control (n=61)
(2) 10 ng/ml LR3 IGF-1 (n=72)

(b) (1) Control (n=38)
(2) 10 ng/ml LR3 IGF-1 (n=32)
(3) 1 ng/ml FSH (n=36)
(4) 10 ng/ml LR3 IGF-1 + 1 ng/ml FSH (n=35)

3.2.2.2. Treatment with FSH in the presence of ascorbic acid

Basic culture medium containing ascorbic acid (control) was prepared as described in 2.2.1.1. The treatment groups were:

(1) Control (n=14)
(2) 10 ng/ml FSH (n=15)
(3) 100 ng/ml FSH (n=32)

3.2.2.3. Treatment with LR3 IGF-1 and recombinant IGF-1 in the presence of ascorbic acid

Basic culture medium containing ascorbic acid (control) was prepared as described in 2.2.1.1. Human recombinant IGF-1 (hrIGF-1) was purchased from Sigma. The treatment groups were:

(1) Control (n=79)
(2) 10 ng/ml LR3 IGF-1 (n=79)
(3) 10 ng/ml hrIGF-1 (n=80)

3.2.3. Measurement of Oestradiol by Double Antibody Radioimmunoassay

Concentrations of oestradiol in unextracted culture media from experiments 3.2.2.2 and 3.2.2.3 were determined by radioimmunoassay as described previously (Webb *et al.*, 1985). Briefly, each standard (100 µl oestradiol 17β; 0-1000 pmol/l [NHS Labs]) was dispensed into assay tubes (Camlab, Cambridge, UK) in quadruplicate. Samples of culture medium (25 µl) were made up to 100 µl using PBS-Gel assay buffer (0.05 M PBS [Sigma] with 0.1% gelatine [Merck] and 0.1% sodium azide [Sigma]), and dispensed in duplicate. A 100 µl aliquot of first antibody (1: 1,500 000 in PBS-Gel) was added to the samples, followed by 100 µl of iodinated steroid (I^{125} oestradiol [NHS Labs]; approximately 10,000 counts per min).The assay was incubated overnight at 4 °C. Equal volumes of normal sheep serum (1:200 in PBS-Gel [SAPU]) and second antibody (sheep anti goat; 1:20 in PBS-Gel [SAPU]) were mixed and 250 µl added to the samples. Following an overnight incubation at 4 °C, 250 µl PBS-Gel containing 1% Tween 20 (Sigma) was added and the tubes were centrifuged (30 min, 3000 rpm, 4 °C). The supernatants were discarded, the tubes drained for 20 min, and the radioactivity in the precipitates counted on a gamma counter (LKB Wallac, 1261 Multigamma). Quantification of oestradiol was performed using AssayZap (Software Phil Taylor 2000, distributed by Biosoft). The sensitivity of the assay was 10 pg/ml. The intra-assay coefficient of variation was <10 %.

3.2.4. Statistical Analyses

Mean follicle diameter and oestradiol production between experimental groups on every second culture day were compared using a one-way ANOVA, with subsequent *t*-tests to allow for individual comparisons between groups.

3.3. RESULTS

3.3.1. Follicular Growth

3.3.1.1. Effect of FSH on follicular growth

Follicles were cultured for 6 days in control medium (n=14) or in medium containing either 10 ng/ml FSH (n=15) or 100 ng/ml FSH (n=32). There was significant follicle growth over 6 days in all groups ($p < 0.01$) (Figure 3.1). FSH did not increase follicle diameter over control levels.

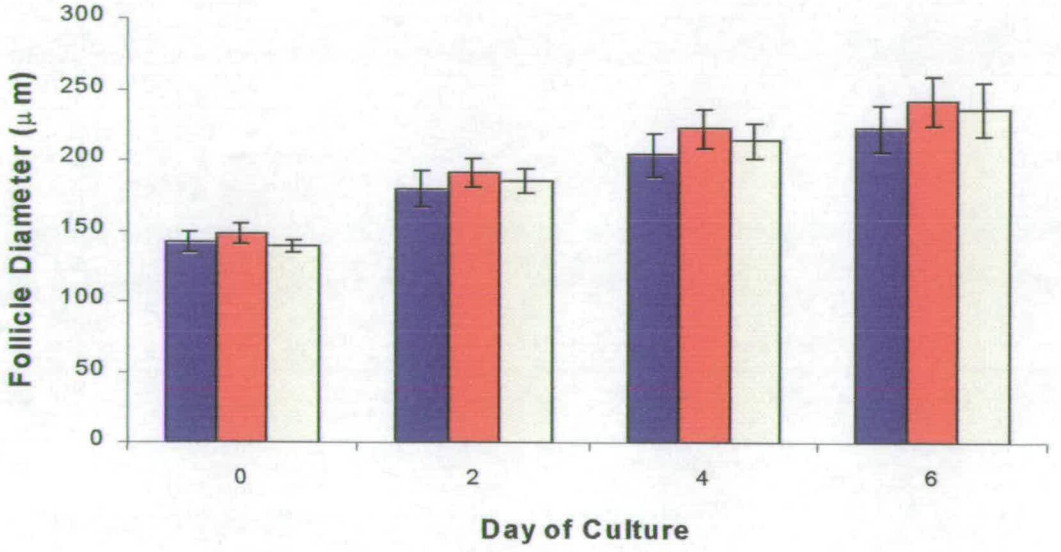
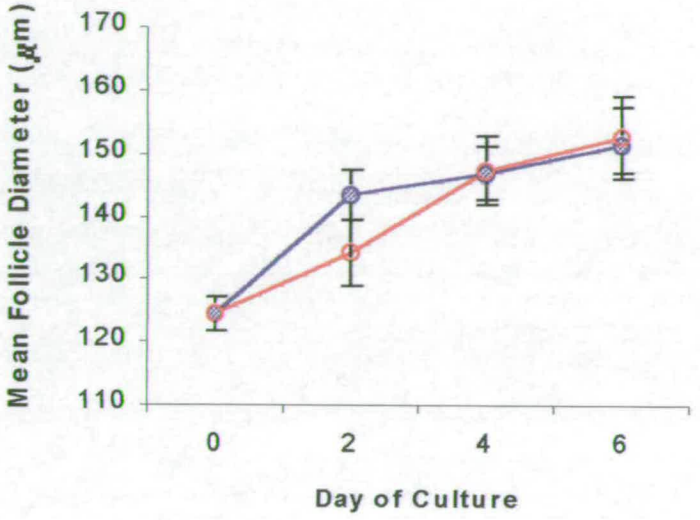


Figure 3.1. Effect of FSH on follicular growth. Growth of preantral follicles in control medium (blue) or in the presence of FSH (red: 10 ng/ml; yellow: 100 ng/ml). Values are mean ± SEM. Growth is significant between Days 0 and 6 within treatment groups ($p < 0.01$).

3.3.1.2. Effect of LR3 IGF-1 on follicular growth

Follicles were cultured for 6 days in the presence (n=72) or absence (n=61) of LR3 IGF-1, with follicle diameters being measured every second day. For analysis of growth in this initial experiment, follicles were separated into two size classes: preantral (<150 μm; n=60), and large preantral (150-200 μm; n=73). As Figure 3.2 illustrates, significant follicular growth occurred over 6 days in the presence and absence of LR3 IGF-1 in both preantral and large preantral follicles ($p < 0.01$). No significant effect of LR3 IGF-1 on follicular growth over control levels was found.

(a)



(b)

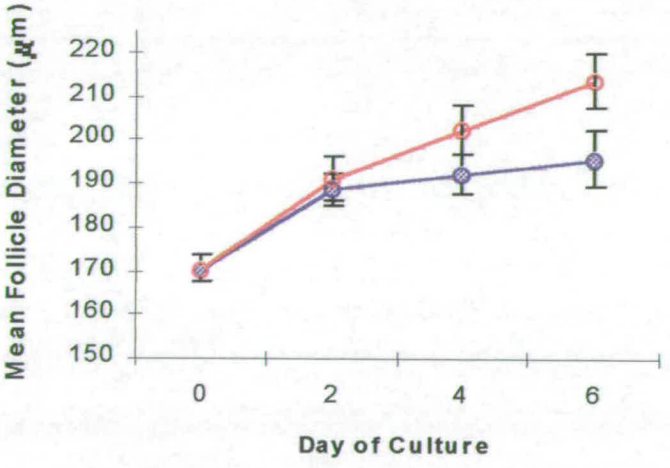


Figure 3.2. Effect of LR3 IGF-1 on follicular growth. Growth of (a) preantral and (b) large preantral follicles in the presence (closed circles) and absence (open circles) of LR3 IGF-1. Values are mean ± SEM. Growth is significant between Days 0 and 6 within both treatment groups ($p < 0.01$).

3.3.1.3. Effect of LR3 IGF-1 and FSH on follicular Growth

Follicles were cultured for 6 days in control medium (n=38) or in the presence of LR3 IGF-1 (n=32), FSH (n=36) or LR3 IGF-1 + FSH (n=35). Significant follicular growth occurred over 6 days in all treatment groups (Figure 3.3) ($p < 0.01$). There was no significant effect of LR3 IGF-1, FSH or LR3 IGF-1+FSH on follicular growth.

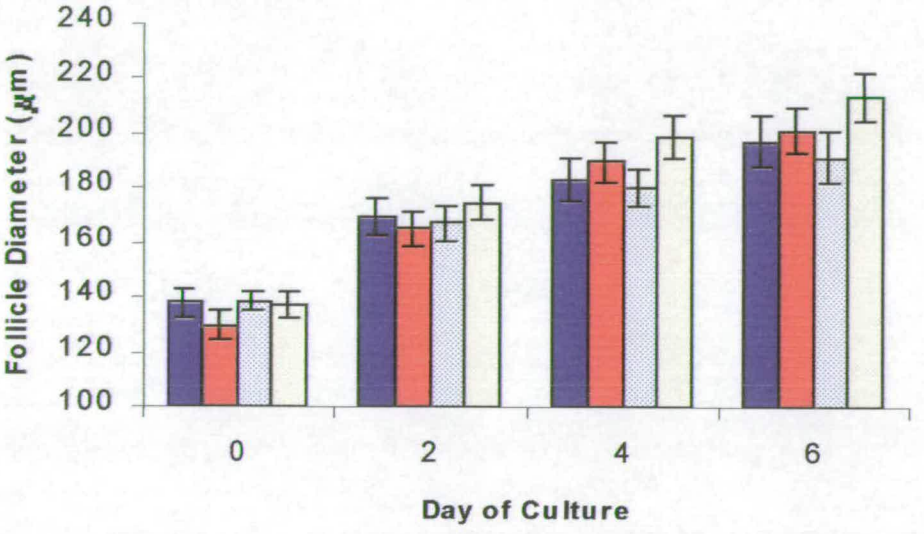


Figure 3.3. Effect of FSH and LR3 IGF-1 on follicular growth. Growth of preantral follicles in control medium (dark blue) or in the presence of LR3 IGF-1 (10 ng/ml, red), FSH (1 ng/ml, light blue) or LR3 IGF-1 + FSH (yellow). Values are mean ± SEM. Growth is significant between Days 0 and 6 within all groups ($p < 0.01$).

3.3.1.4. Effect of recombinant IGF-1 on follicular Growth

Follicles were cultured for 6 days in control medium (n=79) or in medium containing recombinant IGF-1 (n=80) or LR3 IGF-1 (n=79). As illustrated in Figure 3.4, there was significant follicle growth over 6 days in the presence and absence of IGF-1 or LR3 IGF-1 ($p < 0.01$). At the end of culture, there was no significant difference in diameter between control follicles and follicles treated with IGF-1 or LR3 IGF-1.

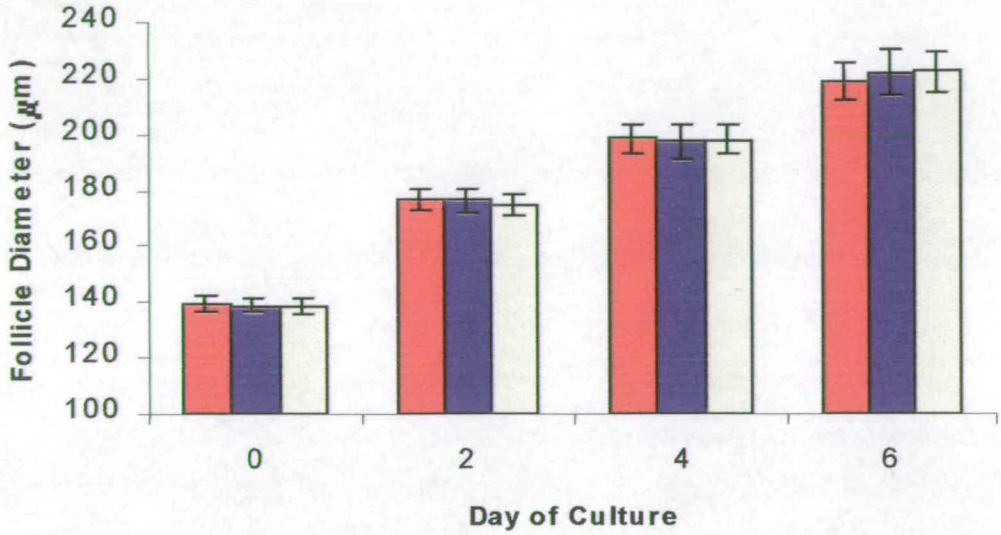


Figure 3.4. Effect of recombinant IGF-1 on follicular growth. Growth of preantral follicles in control medium (blue) or in the presence of recombinant IGF-1 (yellow) or LR3 IGF-1 (red). Values are mean ± SEM. Growth is significant between Days 0 and 6 within treatment groups ($p < 0.01$).

3.3.2. Oestradiol Secretion

3.3.2.1 Effect of IGF-1 and FSH on oestradiol secretion

Medium from follicles cultured in control medium (Day 2, n=43; Day 4, n=37; Day 6, n=41), or in the presence of LR3 IGF-1 (Day 2, n=26; Day 4, n=23; Day 6, n=26), recombinant IGF-1 (Day 2, n=23; Day 4, n=23; Day 6, n=28) or FSH (10 ng/ml: Day 2, n=14; Day 4, n=15; Day 6, n=14; 100 ng/ml: Day 2, n=30; Day 4, n=31; Day 6, n=24) was analysed for oestradiol content by radioimmunoassay. During the 6 day culture period, there was significant ($p< 0.01$) oestradiol secretion by follicles in the presence and absence of LR3 IGF-1, recombinant IGF-1 and FSH (Figures 3.5 and 3.6). There was no significant difference in oestradiol secretion between IGF-1 groups and controls. On Day 2 and 4 of culture, 10 ng/ml and 100 ng/ml FSH stimulated oestradiol secretion ($p< 0.05$) (Figure 3.6). However, only 100 ng/ml FSH maintained this increase by Day 6 ($p< 0.01$).

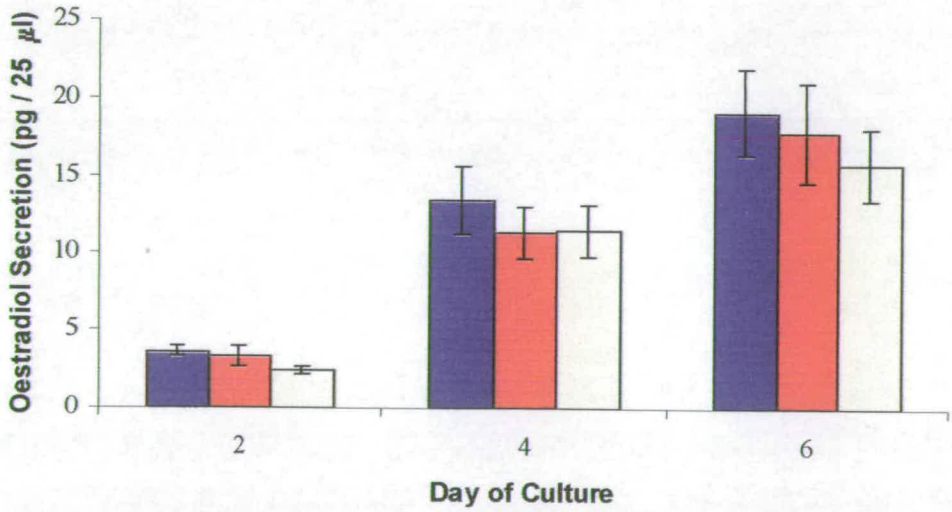


Figure 3.5. Effect of IGF-1 on oestradiol secretion. Oestradiol secretion by preantral follicles cultured in control medium (blue) or in the presence of LR3 IGF-1 (red) or recombinant IGF-1 (yellow). Values are mean ± SEM. Oestradiol secretion is significant between Days 2 and 6 within treatment groups ($p< 0.01$).

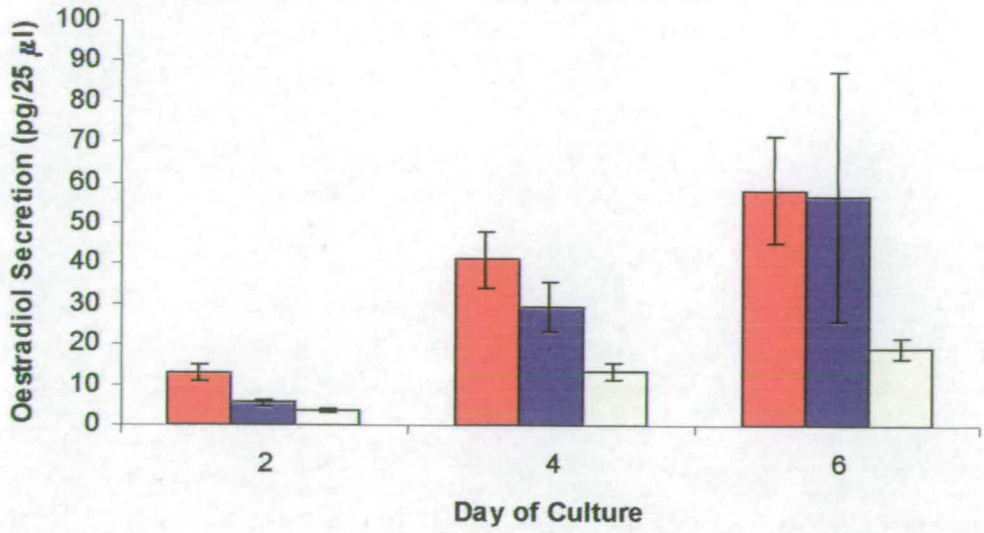


Figure 3.6. Effect of FSH on oestradiol secretion. Oestradiol secretion by preantral follicles cultured in control medium (yellow) or in the presence of 10 ng/ml FSH (blue) or 100 ng/ml FSH (red). Values are mean ± SEM. Oestradiol secretion is significantly increased by FSH (10 ng/ml and 100 ng/ml) from Day 2 ($p < 0.05$).

3.4. DISCUSSION

During the early stages of follicle development *in vitro*, it is important to provide factors in the culture medium which stimulate follicle proliferation and oocyte growth, without inducing precocious differentiation. Development of bovine preantral follicles to a stage where the oocyte can be matured *in vitro* will require a long term culture period, and the demands of the follicle will change according to the stage of development reached.

The role of FSH in the development of rodent preantral follicles has been demonstrated by the finding that such follicles can be efficiently grown *in vitro* only under gonadotrophin stimulation (Nayudu and Osborn, 1992; Eppig and Schroeder, 1989; Cortvrindt *et al.*, 1997). However, it is likely that there is a difference between rodent and bovine species regarding the absolute requirement for FSH for the growth of preantral follicles *in vitro*. The state of differentiation of the granulosa cells may account for this, given that fundamental differences exist between follicles from these species both in terms of follicle size and rate of development. *In vivo*, murine follicles develop from the primordial to the preovulatory stage in approximately 21 days (Pederson, 1970), whereas in cattle and sheep, it is estimated that 4 to 6 months is required for a primordial follicle to reach the Graafian stage (Gosden *et al.*, 1994; Lussier *et al.*, 1994).

Though not a mandatory factor, FSH has been shown to stimulate the growth of bovine and ovine preantral follicles (Hulshof *et al.*, 1995; Wandji *et al.*, 1996; Cecconi *et al.*, 1999; Gutierrez *et al.*, 2000). Ralph (1996) showed that FSH increased follicle diameter in large preantral/early antral (150-210 μm) follicles, but had no effect on small preantral (90-150 μm) follicles. The concentration of FSH used in culture, as well as follicle starting size may account for variation in the effect of FSH within different culture systems. We have shown that physiological concentrations of FSH do not increase follicle diameter over control levels throughout a 6 day culture period. In the study by Ralph (1996), the follicles which were stimulated by FSH were entering the antral phase of development, which is characterised by increased dependence on gonadotrophin support. Cecconi and colleagues (1999) showed that FSH concentrations of 0.01-1 μg/ml stimulated

growth of ovine follicles during a six day culture. However, this system contained serum, as did the system employed by Ralph (1996). Using the serum-free system we have described, Gutierrez *et al.* (2000) showed that a low concentration (1 ng/ml) of FSH stimulated bovine preantral follicle growth during a 28 day culture period. This extended incubation may have allowed sufficient granulosa cell differentiation to occur, thus allowing FSH to have a stimulatory effect.

Granulosa cell proliferation in small bovine follicles has been found to be increased by FSH (Wandji *et al.*, 1996). In contrast, Ralph (1996) reported that an accumulation of intercellular fluid in FSH-treated follicles may have been responsible for increased follicle diameter, rather than an increase in granulosa cell number. This finding questions the usefulness of follicle growth as a representation of physiological development. We have shown here that although FSH does not increase follicle diameter during culture, it significantly increases oestradiol secretion by preantral follicles from Day 2 of culture onwards. This dissociation of follicle growth and oestradiol synthesis confirms previous findings in sheep, human and mouse follicles (Cecconi *et al.*, 1999; Schoot *et al.*, 1992; Boland and Gosden, 1994).

Since no IGF expression has been detected in bovine follicles until after antrum formation, it might be hypothesised that IGFs may play a limited role in preantral follicle development. However, IGF-1 binding has been demonstrated in bovine preantral follicles (Wandji *et al.*, 1992) and type 1 IGF receptor mRNA has been detected in bovine oocytes, granulosa and theca cells from the preantral stage (Armstrong *et al.*, 2000). Therefore, the source of IGF ligand for preantral follicles *in vivo* is probably endocrine rather than paracrine or autocrine. As antral follicle development proceeds, intrafollicular as well as endocrine mechanisms are likely to be involved in IGF regulation of follicular processes (Armstrong *et al.*, 2000). For example, IGF-1 mRNA has been detected at low levels in granulosa cells of bovine antral follicles, with an increase after selection (Schams *et al.*, 1999), and also in theca cells (Gutierrez *et al.*, 1996), indicating that endogenous IGF-1 may be important in the later stages of folliculogenesis, for example in relation to LH responsiveness. This growth factor has also been reported to augment granulosa cell FSH receptor expression in mouse follicles (Zhou *et al.*, 1997). IGF-1 did not

increase follicle growth or oestradiol secretion by preantral follicles in this study. Furthermore, follicle growth was not stimulated by a combination of IGF-1 and FSH. Previously, IGF-1 has been reported to have a stimulatory effect on bovine preantral follicle and oocyte growth in long term culture (Gutierrez *et al.*, 2000). However, that study employed a longer culture period than is reported here. As discussed earlier, as well as allowing a response to FSH, the extended incubation period may have allowed the granulosa cells to differentiate sufficiently to respond to IGF-1 stimulation. Therefore, it is necessary to investigate the stage-specific requirements of the oocyte and follicular cells for IGFs.

In summary, we found no effect of IGF-1 or FSH on follicle growth during a six day culture period. However, increased oestradiol secretion by FSH-treated follicles implies that follicle growth and granulosa cell steroidogenic activity may be differentially regulated. The observed elevation of oestradiol may be due to precocious granulosa cell differentiation, which may be detrimental to the follicle in the early stages of development.

The results presented here demonstrate that preantral follicles can be grown in a serum-free culture system. The following chapters will use this system to further investigate follicle and oocyte development during the early stages of folliculogenesis. Moreover, rather than relying on follicle growth as a indicator of follicle development, we will examine follicle and oocyte morphology *in vitro*. Furthermore, as follicular requirements will change throughout development in a long term culture, it is essential to identify markers of follicle and oocyte development.

Chapter 4

Matrix Metalloproteinases and their Endogenous Inhibitors: Markers for Follicle Health

4.1. INTRODUCTION

Compared to the current knowledge of regulation of follicular development by hormones and growth factors, relatively little is known of the cellular biology of developing follicles. The follicular basement membrane is a specialised extracellular matrix which influences epithelial cell migration, proliferation and differentiation, and can selectively retard the passage of molecules from one side of the basement membrane to the other (Rodgers *et al.*, 2000). Previously, culture systems for bovine preantral follicles have resulted in the loss of theca cells (Ralph, 1996; Gutierrez *et al.*, 2000) or rupture of the basement membrane (Gutierrez *et al.*, 2000) in some follicles.

During follicular growth, turnover and reconstruction of the basement membrane is facilitated by matrix metalloproteinases (MMPs). These are zinc and calcium-dependent enzymes that are capable of degrading the protein components of the extracellular matrix (ECM) (Smith *et al.*, 1997). MMPs are regulated by tissue inhibitors of metalloproteinases (TIMPs), and are responsible for the reconstruction of the basement membrane at the time of ovulation, and during corpus luteum formation (Zhao and Luck, 1996). The MMPs involved in the breakdown of collagen IV (a major constituent of the basement membrane) are the gelatinases MMP-2 and MMP-9. The main follicular sources of MMPs and TIMPs are thought to be the granulosa and theca cells (Zhao and Luck, 1996; Smith *et al.*, 1994), but TIMP protein has also been detected in the oocyte (Bagavandoss, 1998).

The role of growth factors in control of follicular cell proliferation has been studied extensively. As discussed in Chapter 1, growth factors are often secreted constitutively and sequestered in the ECM in an inactive form or in association with specific binding proteins. Liberation of these factors by proteolysis of the ECM is a key process in regulation of availability of growth factors and their activities within developing follicles. MMPs have been reported to function as IGFBP-degrading proteinases, both *in vitro* and *in vivo* (Fowlkes *et al.*, 1994a; Fowlkes *et al.*, 1994b; Thrailkill *et al.*, 1995). Therefore, MMPs, beyond their previously described roles as extracellular matrix degrading enzymes, may also exert effects on cellular growth and differentiation via degradation of growth factor binding proteins.

Adequate basement membrane turnover is essential for follicular growth and survival. However, previous culture systems for bovine preantral follicles have resulted in the loss of theca cells (Gutierrez *et al.*, 2000; Ralph, 1996) or rupture of the basement membrane (Gutierrez *et al.*, 2000) in some follicles. Therefore, factors involved in basement membrane remodelling may be useful indicators of follicular health. The aims of this chapter were (1) to determine whether MMPs are secreted by bovine follicles *in vivo*; (2) to identify secretion of MMPs and TIMPs by preantral follicles *in vitro*, and using growth and morphological data, assess these factors as markers of follicular development; and (3) to determine whether IGF-1 affects MMP and TIMP secretion *in vitro*.

4.2. MATERIALS AND METHODS

4.2.1. Collection of Follicular Fluid

Bovine ovaries were collected as described in 2.1.2. Follicular fluid was aspirated from antral follicles (0.65 mm-2.5 cm; n=6) present on the surface of the ovaries using syringes fitted with 25 G needles. Follicular fluid aliquots were stored at -20 °C for subsequent MMP analysis.

4.2.2. Follicle Isolation and Culture

Preantral follicles (< 200 μm) were isolated from bovine cortical slices and cultured individually for 6 days as described in chapter 2. Half of the culture medium was replaced every second day, and this conditioned medium was stored at -20 °C for subsequent MMP/TIMP analysis.

4.2.3. Treatments

Culture medium was prepared as described in 2.2.1. For the treatment group, 10 ng/ml of LR3 IGF-1 (Gropep) was added to the control medium.

4.2.4. Detection of Secretion of MMPs and TIMPs

4.2.4.1. Detection of secretion of MMP-2 and MMP-9 by gelatin zymography

Cuture medium was collected every second day, and MMP/TIMP analysis done at random time points. MMP-2 and MMP-9 activities were detected in follicular fluid (n=6) and culture medium (n=52) by gelatin zymography as described previously (Riley *et al.*, 1999). In brief, 100 μl samples of culture medium or follicular fluid were dialysed against distilled H_2O in small tube-o-dialysers (Chemicon International, London, UK) before being lyophilised and reconstituted in 7.5 μl 0.1% SDS in H_2O.

Samples were separated by SDS-PAGE using 7.5% gels containing 1mg/ml gelatin on a minigel apparatus (Bio-Rad, Hemel Hempstead, UK). Gels were washed (2 x 15 min, 2.5% (v/v) Triton X-100; 2x2 min TBS x10) then incubated overnight at 37 °C in digestion buffer (50 mM Tris; 0.2 M NaCl; 5 mM $CaCl_2$, 1 μM $ZnCl_2$; 0.02% (v/v)Brij-35) Gels were stained for 3 h at 23 °C (0.5% Coomassie Blue R250 in 30% methanol / 10% glacial acetic acid in H_2O), then destained (staining solution with Coomassie Blue omitted). This reveals clear bands where gelatin has been degraded by gelatinase activity. MMP-2 and MMP-9 were identified by comparison with molecular weight markers and control standards of human amniotic fluid collected during labour at term (Riley *et al.*, 1999).

4.2.4.2. Detection of secretion of TIMP-1 and TIMP-2 by reverse zymography

TIMP activity was detected in culture medium (n=24) using a commercial kit (University Technologies), as described previously (Riley *et al.*, 1999). Samples were lyophilised and reconstituted as before and separated by molecular weight by PAGE, using 12% gels containing gelatin (1 mg/ml) and a preparation of MMP-2 (conditioned medium from BHK-21 cells that constitutively express MMP-2; University Technologies Inc, Calgary, Canada). The gels were washed (50 mM Tris, 5 mM $CaCl_2$, 2.5% (v/v) Triton X-100) for 2.5 h at 23 °C, then incubated in digestion buffer (50 mM Tris, 5 mM $CaCl_2$) overnight at 37 °C. After staining and destaining (as in zymography), TIMPs were represented by discrete dark bands on the gel, where inhibition of MMP-induced degradation of the gelatin substrate has occurred. TIMPs were identified by comparison with molecular weight markers and control standards of conditioned medium containing mouse TIMP-1, -2 and -3 expressed by transfected BHK cells (University Technologies).

4.2.5. Histological Assessment

At the end of the culture period, follicles were fixed and processed for histology as described in chapter 2. Granulosa cell death was measured by counting the number of pyknotic cells and expressing them as a percentage of the total number of granulosa

cells: atretic follicles were defined as those with >5% pyknotic nuclei (Byskov, 1974). Theca cell health was assessed in two ways: (a) by the level of pyknosis, as defined above, and (b) by the number of layers present, with < 3 layers indicating theca degeneration.

4.2.6. Statistical Analyses

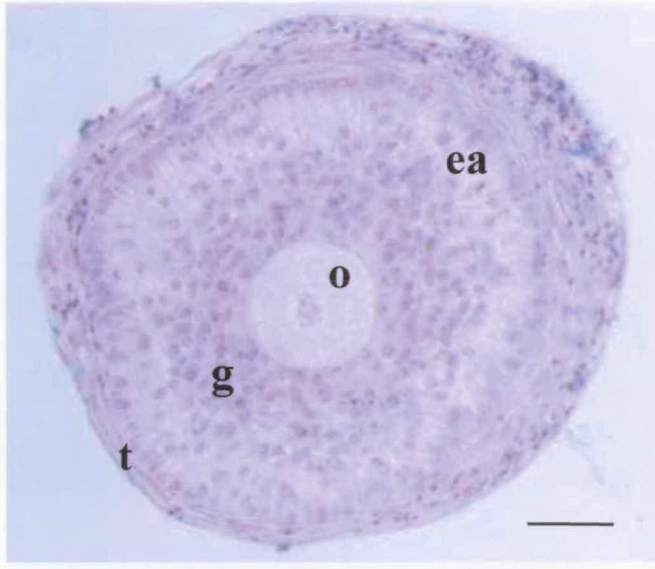
With probability estimates for the occurrence of MMPs and TIMPs having been calculated, Baye's Theorem (Clarke and Cooke, 1992) was applied in order to estimate the probability of granulosa or theca cell health, given the presence of MMPs and TIMPs. Comparison of MMP and TIMP secretion between IGF-1-treated and control follicles was performed using the 2-proportions test on MINITAB.

4.3. RESULTS

4.3.1. Histological Assessment

Histological observations were made using freshly isolated follicles (n=10), IGF-1 treated follicles (n=24) and control follicles (n=22). These results will be discussed in detail in chapter 6. For the purposes of the present study, follicular granulosa and theca cells were termed either healthy or degenerate according to the criteria set out in 4.2.5. Examples of follicles with healthy and degenerating somatic cells are shown in Figure 4.1.

(a)



(b)

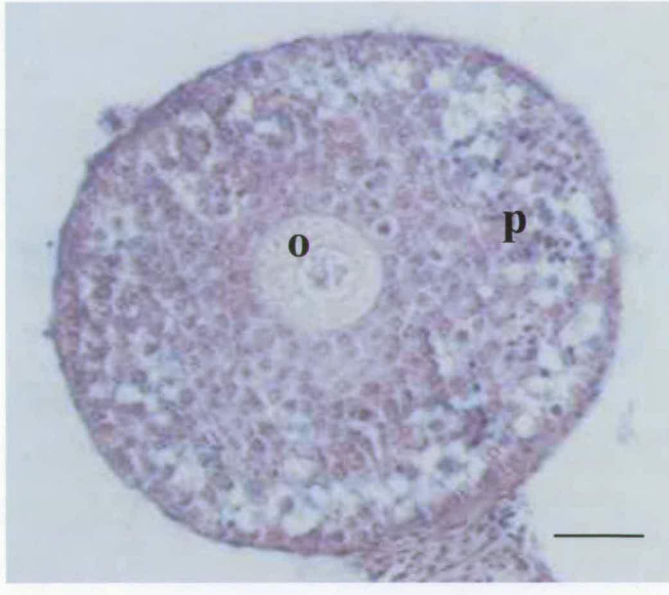


Figure 4.1. Histological sections representing healthy and degenerating follicles. **(a)** a follicle with a healthy oocyte (o), even granulosa cells (g) with spacing that may be indicative of an early antrum (ea), and a differentiated theca layer (t); **(b)** a follicle with pyknotic granulosa cells (p) and degenerated theca cells. Bar = 50 μm.

4.3.2. Secretion of MMP-2, MMP-9, TIMP-1 and TIMP-2 Activities

4.3.2.1. MMP secretion in vivo

In this preliminary experiment, follicular fluid from 6 follicles (0.65 mm-2.5 cm diameter) was analysed for gelatinase activity. There was abundant MMP-2 activity and small amounts of MMP-9 activity in 5 out of 6 samples. A representative zymogram is shown in Figure 4.2.

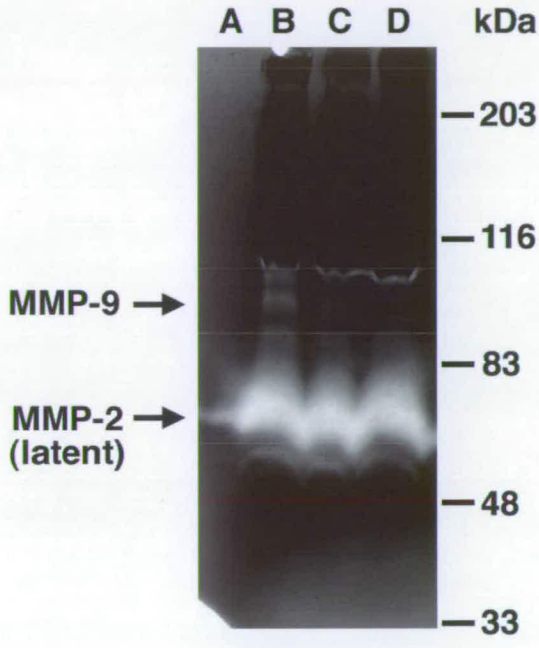


Figure 4.2. Representative gelatin zymogram showing gelatinase (MMP) activity secreted in 4 samples of follicular fluid from **(A)** a 0.65 mm follicle, **(B)** a 3 mm follicle, **(C)** a 9 mm follicle and **(D)** a 2.5 cm follicle.

4.3.2.2. Effect of growth status on MMP secretion in vitro

In the first *in vitro* experiment, culture medium from 20 follicles was analysed for gelatinase activity. Of these samples, 12 were from follicles observed to be growing, and 8 were from follicles which remained static prior to collection of medium. MMP-2 activity was detected in all samples. However, only 50% of the static follicles and 66.7% of the growing follicles, respectively, secreted MMP-9 into the culture medium. The preliminary gel indicated that there may have been an increase in MMP activity in growing follicles, as illustrated in Figure 4.3. However, after analysis of all samples, no significant correlation between MMP-2 or MMP-9 secretion and growth status was found.

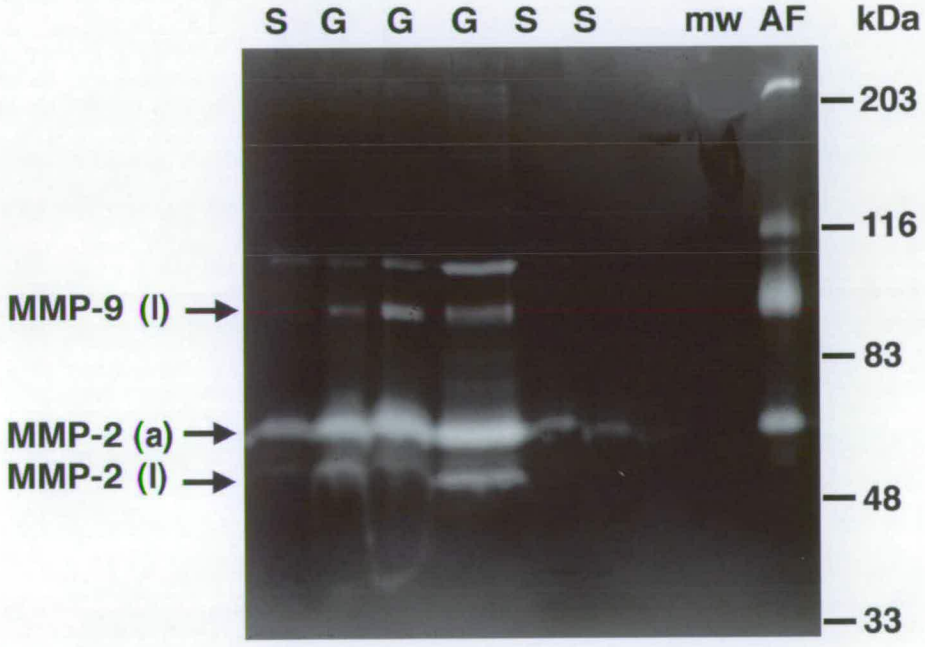


Figure 4.3. Representative gelatin zymogram showing gelatinase (MMP) activity secreted in samples of culture medium from static (S) and growing (G) bovine follicles. Human amniotic fluid was used as a positive control (AF). Molecular weight markers (mw) are as indicated (kDa).

4.3.2.3. Effect of follicle morphology on MMP and TIMP secretion in vitro

Culture medium from 32 follicles was analysed for gelatinase activity, and 24 samples were analysed for the presence of TIMPs. Examples of a zymogram demonstrating MMP-2 and MMP-9 activity, and a reverse zymogram showing TIMP-1 and TIMP-2 secretion by follicles into the culture medium are shown in Figure 4.4. MMP-2 (72 kDa) was found to be released by 87.5% of follicles, whereas MMP-9 (92 kDa) was secreted by 62.5% of follicles. Comparisons between zymographic analysis and morphology (Table 4.1) showed that if MMP-9 was secreted during culture, the probability of these follicles having healthy (as determined by level of pyknosis) granulosa cells at the end of the culture period was 0.85. The probability of theca cell health (defined by the number of layers) was found to be 0.60. When TIMP-1 (28 kDa) was released *in vitro*, (58% follicles), there was a probability of 0.79 that the follicles would have healthy granulosa or theca cells. When TIMP-2 (21 kDa) was produced (75% follicles), the probability of the follicles having healthy granulosa or theca cells was 0.78 and 0.67, respectively. There was no significant difference in MMP or TIMP secretion between IGF-1-treated and control follicles (Tables 4.2a and 4.2b).

Table 4.1. Effect of follicle morphology on MMP and TIMP secretion

Secreted Factor Present	Probability of Granulosa Cell Health	Probability of Theca Cell Health
MMP-9	0.85	0.60
TIMP-1	0.79	0.79
TIMP-2	0.78	0.67

Table 4.2a. Effect of IGF-1 on MMP secretion

	MMP-2 Secretion (% Follicles)	MMP-9 Secretion (% Follicles)
IGF-1 (n=17)	94.1	52.9
Control (n=13)	76.9	61.5

Table 4.2b. Effect of IGF-1 on TIMP secretion

	TIMP-1 Secretion (% Follicles)	TIMP-2 Secretion (% Follicles)
IGF-1 (n=8)	50	62.5
Control (n=12)	66.7	83.3

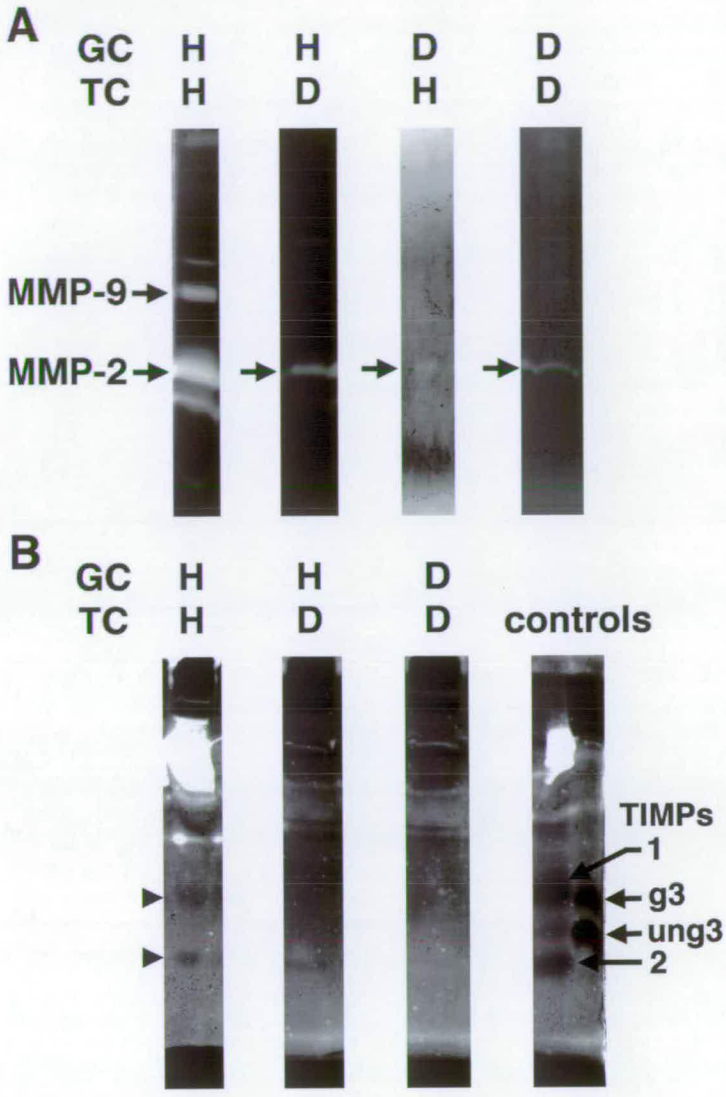


Figure 4.4. Representative gelatin zymogram (A) and reverse zymogram (B) showing gelatinase (MMP) and TIMP activities secreted in samples of culture medium. MMP-2 and MMP -9 or TIMP-1 and TIMP-2 presence is indicated in four follicles with granulosa cells (GC) that are healthy (H) or degenerate (D) and theca cells (TC) that are healthy (H) or degenerate (D).

4.4. DISCUSSION

Regarding the preantral and early antral stages of follicular development, very little is known concerning the contribution of the various MMPs and associated regulators to follicular development. We have demonstrated that MMP-2 and MMP-9 are secreted into follicular fluid, and when MMP-9 and TIMPs are secreted by preantral follicles *in vitro*, a follicle has a higher probability of being healthy (as determined by level of pyknosis in granulosa cells and number of theca layers) at the end of the culture period. However, the secretion of MMPs was not correlated to follicle growth rate. There was also no significant difference in MMP and TIMP secretion by IGF-1-treated and control follicles. In the previous chapter, it was demonstrated that IGF-1 had no effect on follicular growth (as measured by follicle diameter) in our culture system. As will be discussed in chapter 6, we have also found no evidence of a difference between IGF-1-treated and control follicles in terms of somatic cell health.

Several studies have investigated the distribution of MMPs and TIMPs at different stages of follicular development. In the ovary of neonatal rats, activity of MMP-2, but not MMP-9, has been detected, with visualisation of TIMP-1 in the oocyte (Bagavandoss, 1998). In PMSG-primed ovaries of rats, and also mice, MMP-2 is detected in the granulosa and theca cells, MMP-9 is restricted to thecal and interstitial cells, and TIMP-1 is located in blood vessels and theca cells (Hagglund *et al.*, 1999; Bagavandoss, 1998; Reich *et al.*, 1991). In addition, MMP-9 is restricted to the theca cell layers in goat follicles of less than 3 mm (Garcia *et al.*, 1997), and has been detected in differentiating granulosa cells in culture (Zhao and Luck, 1996) and subsequently in the developing corpus luteum in rats (Bagavandoss, 1998). Collectively, the results of these studies implicate the granulosa and theca cells as sources of MMPs and TIMPs during follicular development.

In vitro, secretion of MMP-2 and MMP-9 from bovine thecal cells has been observed to increase in response to LH (Smith *et al.*, 1997). This observation may imply that theca cells have a role in remodelling the basement membrane, or that the remodelling of vascular tissue present within the culture may be under gonadotrophin control. The regulation of MMP activity is complex and may be controlled at the level

of transcription by growth factors, cytokines and hormones, with subsequent activation of secreted proforms, and inhibition by TIMPs (Hagglund *et al.*, 1999). The mechanisms controlling remodelling at the gonadotrophin-independent stages of follicle development are unknown; thus, further investigations are required. MMPs and TIMPs also may be regulated depending on their specific spatial and temporal functions. The distribution of MMP-9 in the steroidogenic cells of the theca layer, interstitium, and corpus luteum as well as its absence in the neonatal ovary (Bagavandoss, 1998) and the timing of expression (Chaffin and Stouffer, 1999), suggest a role in the remodelling and vascularisation associated with corpus luteum formation. The detection of MMP-2 and MMP-9 during our study in cattle suggests a role for these enzymes at a much earlier stage than, to our knowledge, has been studied previously, and that MMP-9 and TIMPs can now be used as non-invasive markers for assessing follicular quality *in vitro*.

TIMPs have been implicated in other processes involving cell growth. For example, TIMP-1 has been associated with erythroid potentiating activity (Docherty *et al.*, 1985) and TIMPs -1 and -2 have growth factor-like activities in some cell types (Hayakawa *et al.*, 1994). The increased probability of follicular health when TIMPs were present in our study agrees with this function. Results of other reports suggest that TIMP-1 is a facilitator of steroidogenesis (Duncan *et al.*, 1996; Nothnick and Curry, 1996; Boujrad *et al.*, 1995). Differences in TIMP activity between atretic and non-atretic follicles during the late preantral and early antral stage may be a marker for the shift in steroidogenic capability and future selection of healthy follicles at this stage of development. The significance of the location of TIMP-1 in the oocyte (Bagavandoss, 1998) is unclear, and during the early stages of folliculogenesis, a potential function for TIMPs as oocyte growth factors remains uninvestigated. Future experiments should assess if the correlation between MMP and TIMP secretion with follicle quality *in vitro* also applies to oocyte health and development. This will allow us to better understand interactions between the oocyte and somatic cells regarding the coordination of oogenesis and folliculogenesis.

In our study, follicles that did not maintain a differentiated thecal layer *in vitro* tended to be less healthy overall than follicles with thick, non-pyknotic theca cell

layers. Maintaining interplay between the oocyte and the surrounding somatic cells is vital to achieve normal folliculogenesis. Growth differentiation factor-9 (GDF-9), which is an oocyte-derived factor from the transforming growth factor-β superfamily, is required for sustaining follicular growth and differentiation after the primary (i.e. one-layer) follicular stage (Dong *et al.*, 1996). Studies using knockout mice (Elvin *et al.*, 1999; Dong *et al.*, 1996) have shown that in the absence of GDF-9, follicles are incompetent to emit a signal that recruits theca cell precursors to surround the basement membrane (Elvin *et al.*, 1999). Our observations confirm the importance of maintaining healthy theca cells and their connections with the granulosa layers in bovine follicle culture, and also support a role for these cell types in basement membrane remodelling during the early stages of folliculogenesis. Having said this, murine oocytes can obtain developmental competence and be fertilised *in vitro* in the absence of theca cells (Eppig and Schroeder, 1989; Eppig and O'Brien, 1996). However, there may be species differences regarding the requirement of the oocyte for theca cell factors *in vitro*. Conversely, the presence of theca cells may further enhance oocyte developmental competence in rodent systems.

Further evidence for the role of theca cells in influencing follicular development is provided by recent studies in sheep (Wilson *et al.*, 2001; Souza *et al.*, 2001). Bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, have been shown to be expressed in theca cells, and BMP receptors have been found in granulosa cells and oocytes of normal cycling rats (Shimasaki *et al.*, 1999). Interestingly, the prolific Booroola phenotype is associated with a mutation in the BMP receptor type 1B (BMPR1B) gene (Wilson *et al.*, 2001; Souza *et al.*, 2001). Hence a mutation in the BMP signalling system, which involves interaction between the oocyte and follicular cells, may contribute to the precocious follicle differentiation and increased ovulation rate observed in Booroola animals.

In conclusion, we have detected MMP and TIMP activity *in vitro* and identified MMP-9, TIMP-1, and TIMP-2 as markers of follicular health. Elucidation regarding the action of MMPs and TIMPs and the role of growth factors during follicle growth and antrum formation in an extended culture will provide insight into the complex processes involved in follicle and oocyte growth and development.

Improving our knowledge of the interactions between follicular cells and the extracellular matrix is necessary for maintaining the integrity of preantral follicles *in vitro*, thus sustaining follicular viability.

Chapter 5

The Effect of Ascorbic Acid on Preantral Follicle Development *In Vitro*

5.1. INTRODUCTION

In the previous chapter, MMP-9 and TIMPs, which are involved in basement membrane remodelling, were identified as indicators of follicular health using a serum-free culture system. This led to further investigation into factors that affect basement membrane turnover. One such factor is ascorbic acid (review: Luck *et al.*, 1995). In the present chapter, the culture period is extended from 6 to 12 days, and the effect of ascorbic acid on the development of preantral follicles *in vitro* is investigated.

Ascorbic acid, a dietary requirement for primates and a few other mammals, has been implicated in several processes associated with follicular and luteal development. It is widely distributed in animal tissues, with the highest concentrations being found in the pituitary, adrenal gland and gonads (Luck *et al.*, 1995). Ascorbic acid accumulates in granulosa cells, theca interna, luteal cells, and in oocytes (Kramer *et al.*, 1933; Hoch-Ligeti and Bourne 1948; Deane, 1952). Acting as an antioxidant, ascorbic acid has been implicated in the processes of hormone secretion, gonadal tissue remodelling and apoptosis (Luck *et al.*, 1995). Adequate turnover of components of the follicular ECM is essential for normal follicle growth, follicle repair after ovulation (Himeno *et al.*, 1984), and subsequent corpus luteum development (Luck and Zhao, 1993). In species with large follicles such as humans and cattle, there is a high demand for collagen IV (a major constituent of the basement membrane). Ascorbic acid has been implicated in the biosynthesis of collagen, both at the gene level (Padh, 1991) and during processing of the protein (Pinnell, 1985; Hulmes, 1992). As discussed in the previous chapter, the enzymes involved in the turnover of collagen during follicle growth are the MMPs which are regulated by TIMPs. Ascorbic acid may act to regulate these factors (Pfeffer *et al.*, 1998; Murray *et al.*, 2001).

The presence of high concentrations of ascorbic acid in endocrine tissues is thought to be important for the production of steroid hormones (Tsuji *et al.*, 1989). In cows, ascorbic acid levels in follicular fluid tend to be higher during the early part of the cycle (days 1-10 after ovulation) (Wise, 1987). This sequestration of ascorbic acid may be to allow rapid expansion of the follicle or to facilitate post-ovulatory

steroidogenesis (Luck *et al.*, 1995). In contrast, it has been suggested that ascorbic acid at high concentrations inhibits steroidogenesis (Sanyal and Datta, 1979). Any effect of ascorbic acid on hormone synthesis during early follicle development remains to be clarified.

The role of ascorbic acid as a free radical scavenger is well understood. It is thought that follicular atresia is initiated as a consequence of inadequate protection of maturing granulosa cells from the damaging effects of reactive oxygen species (Tilly and Tilly, 1995). Oxidants induce apoptosis in cultured granulosa cells, and addition of FSH or antioxidants such as ascorbic acid inhibits this response (Tilly and Tilly, 1995). In addition, addition of serum during culture reduces follicular apoptosis (McGee *et al.*, 1997). As well as follicular atresia, ascorbic acid deficiency causes premature resumption of meiosis and oocyte destruction (Kramer *et al.*, 1933).

The present study aims to determine the effects of ascorbic acid on bovine preantral and early antral follicle development *in vitro*. Follicle growth and basement membrane integrity are assessed during the culture period, and the effect of ascorbic acid on follicular morphology, MMP production, oestradiol secretion and follicular cell death are investigated. In addition, the effect of serum on follicle growth and health is assessed.

5.2. MATERIALS AND METHODS

5.2.1. Follicle Isolation and Culture

Preantral follicles were isolated as described in chapter 2, and cultured for 6, 10 or 12 days as described below.

5.2.2. Treatments

For the control group, preantral follicles were cultured individually for 12 days in medium described in 2.2.1. It should be noted that McCoys 5a medium already contains 0.5 μg/ml ascorbic acid in its standard composition. However, a concentration of 50 μg/ml has been reported to be effective in increasing follicle survival in mouse follicle culture (Murray *et al.*, 2001). For the first treatment group, 50 μg/ml of L-ascorbic acid, sodium salt (Sigma) was added to the control medium and follicles were cultured for 12 days. The effect of foetal bovine serum (FBS [Sigma], 10%) on follicles from Day 6 of culture onwards was also investigated, with addition of FBS to follicles in either ascorbic acid-treated or control groups. In addition, FBS was added to control or ascorbic acid-treated medium on Day 0 and follicles were cultured for 10 days. These treatment groups are illustrated in Figure 5.1. In addition, follicles were cultured for 6 days in control medium (n=22) or ascorbic acid medium (n=10) in the absence of serum, and these follicles were fixed for histological analysis. Follicle diameters were measured under the dissection microscope every second day, and follicles which maintained their 3D morphology were defined as intact. Half of the medium was replaced every second day, and this conditioned medium was stored at -20 °C for subsequent MMP analysis, or for analysis of oestradiol content.

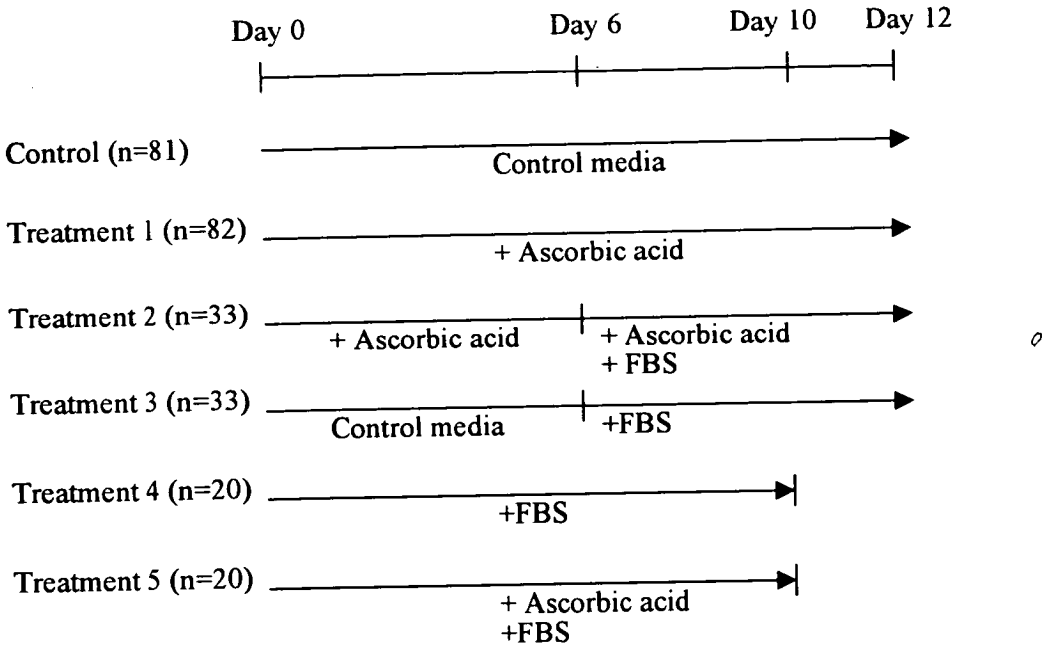


Figure 5.1. Ascorbic acid and serum treatment groups

5.2.3. Histological Assessment

Control follicles (n=22) and follicles treated with ascorbic acid (n=10) were fixed on Day 6 and processed for histological analysis as described in chapter 2. In addition, a proportion of follicles treated with ascorbic acid (n=11) were fixed on Day 12 and processed for histology.

5.2.4. Detection of Apoptotic Cell Death in Follicle Wholemounts by TUNEL

At the end of the 12 Day culture period, follicles were transferred to 24 well culture plates (Corning Costar) and washed (10 min, PBS, 37 °C). Cultured follicles (n=35) and freshly isolated follicles (n=8) were permeabilised and prefixed (40 min, 0.5% Triton X-100, 0.25% paraformaldehyde (PF) in PBS at 37 °C) then fixed (30 min, 4% PF in PBS, RT) washed in PBS (2x10 min) and stored (0.02% NaN_3 in PBS, 4 °C). Follicles were washed and equilibrated to RT with PBS (20 min; 2x10 min). Samples were then incubated with proteinase K (Roche Diagnostics, Lewes, UK) (40 min, 17.1 μg/ml in PBS, 37 °C), refixed at RT (20 min, 3% PF in PBS) and washed (10

min, 0.01% Triton X-100 in PBS; 2x10 min PBS). A preincubation with TdT buffer (10 min, 30 mM Tris-HCl pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) was carried out before incubation with TUNEL reaction mixture (2.5 h, 37 °C in dark) from an In Situ Cell Death Detection Kit, Fluorescein (Roche) which was prepared according to the manufacturer's instructions.

Follicles were washed in PBS (2x10 min, in dark), and incubated with propidium iodide (1 h, 2.5 µg/ml, RT) in bovine pancreatic RNase A (Roche). Samples were washed (20 min, 0.01% Triton X-100 in PBS; 2x10 min PBS) and equilibrated in 50% Vectashield (Vector Laboratories Ltd, Southgate, UK) overnight at 4 °C. Follicles were mounted in 100% Vectashield on concave microscope slides (TAAB Laboratories Ltd, Aldermaston, UK) and stored at 4 °C in the dark.

Follicles were analysed for fluorescein dUTP incorporation using the Leica TCSNT Confocal system (Leica). Using a 63x water corrected PL APO lens, a single scan was taken through the centre of each follicle as determined by central positioning of the propidium iodide stained germinal vesicle in the oocyte (if the oocyte was not visible, the largest cross-section was used). Simultaneous scans at 488 nm (the green channel which shows any TUNEL labelled DNA) and 568 nm (the red channel which shows propidium iodide stained nuclear material) were taken. The number of TUNEL labelled cells (granulosa and theca) and the total number of cells in each section were counted. Due to substantial loss of theca cells in groups treated with serum, the effect of ascorbic acid on theca cell death was analysed in the serum-free groups only.

5.2.5. Detection of MMP-2 and MMP-9 in Culture Medium

Gelatinase activity was detected in serum-free medium (n=24) on Day 8 of culture as described in 4.2.4.1. MMP activities as detected by zymography were measured by transmission densitometry (G-700 Densitometer; BioRad). Relative intensities were derived from zymogram gels by comparison to parallel background readings of equal area and calculated using dedicated software (Quantity One; BioRad). Densitometric readings were only compared with another gel examined under precisely same conditions (i.e. the same electrophoresis run and identical buffers, stains and

incubation periods).

5.2.6. Detection of Oestradiol in Culture Medium

Concentrations of oestradiol in unextracted serum-free culture media from follicles cultured with (Day, 2, 4, 6, 8, 10, 12: n=42, 35, 17, 31, 41, 39) or without (Day, 2, 4, 6, 8, 10, 12: n=45, 30, 28, 36, 32, 34) ascorbic acid were determined every second day of culture by radioimmunoassay, as described in 3.2.3. The sensitivity of the assay was 36 pg/ml. The intra- and inter-assay coefficients of variation were 4.0% and 4.8%, respectively.

5.2.7. Statistical Analyses

Mean percentage increase in follicle diameter in the 6 experimental groups on Day 8, and the four experimental groups on Day 12 were compared using a one-way ANOVA, with subsequent *t*-tests to allow for individual comparisons between groups. Within each group, correlations were obtained between percentage increase in follicle diameter and the percentage of follicles which remained intact during culture. In addition, the proportion of follicles remaining intact on every second day of culture were compared using a 2-proportions test. Follicles fixed for histological assessment on Day 6 were analysed for differences in the % of follicles containing degenerate granulosa cells (as assessed as having >5% pyknotic nuclei) by a 2-proportions test. In addition, oocyte degeneration on Day 6 and Day 12 in follicles cultured with ascorbic acid was assessed by a 2-proportions test. Follicles cultured for 12 days were assessed for granulosa and theca cell death by TUNEL. The percentage of labelled granulosa cells were compared between the four groups by ANOVA. In addition, the number of labelled granulosa cells as a proportion of the total number of cells in each treatment group were compared using chi-square analysis. The effect of ascorbic acid on theca cell death was analysed in the serum-free groups using a 2-sample *t*-test. The number of healthy oocytes as a proportion of the total number of oocytes analysed in ascorbic acid-treated follicles on Day 6 and Day 12 of culture were compared using a

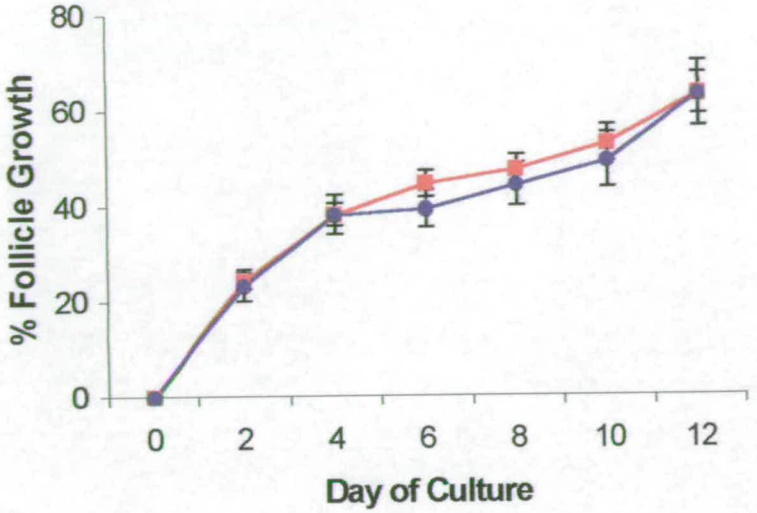
2-proportions test. Oestradiol secretion was measured in serum-free control and ascorbic acid-treated follicles and values compared using ANOVA and subsequent 2-sample *t*-tests. The MMP densitometry data was analysed using a 2-sample *t*-test.

5.3. RESULTS

5.3.1. Follicular Growth

Follicles were cultured for 12 days in the presence (n=82) or absence (n=81) of ascorbic acid in serum-free medium. In addition, follicles were cultured in the presence of FBS which was added on Day 0 (n=40) or Day 6 (n=66) of culture in the presence or absence of ascorbic acid. There was significant follicle growth in all treatment groups ($p< 0.01$) (Figure 5.2). Follicle cultures containing serum from Day 0 were terminated at Day 10 due to an increased occurrence of follicle rupture. As a result of follicle loss in these groups, only measurements up until Day 8 of culture were used for measurement of follicle growth (Figure 5.2b). On Day 8 there was a significant difference in percentage follicle growth between all treatment groups ($p< 0.05$). Follicles cultured in serum (with and without ascorbic acid) from the start of culture had a higher percentage increase in follicle diameter than serum-free controls by Day 8 ($p< 0.05$). However, serum alone did not significantly increase percentage increase in follicle diameter compared to follicles treated with ascorbic acid in serum-free medium. When FBS was added to follicles on Day 6, there was no difference in percentage follicle growth by Day 12 compared to control or ascorbic acid-treated follicles in the absence of serum.

(a)



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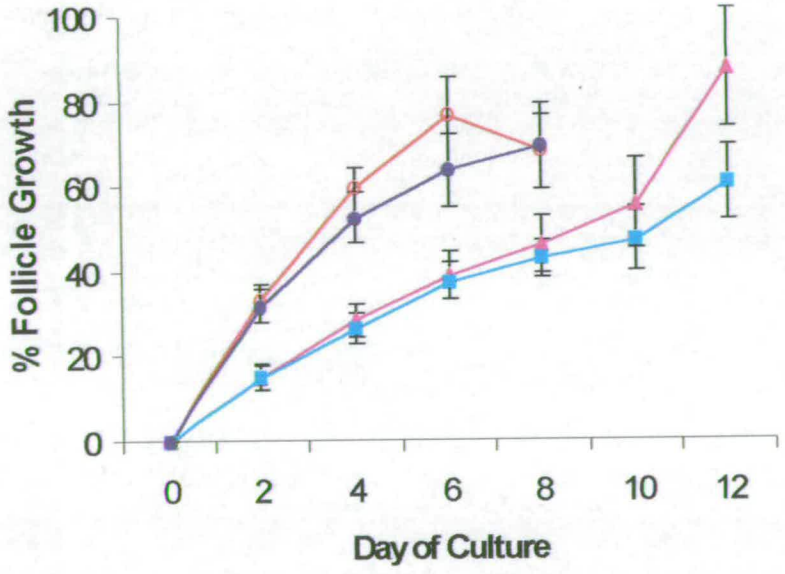
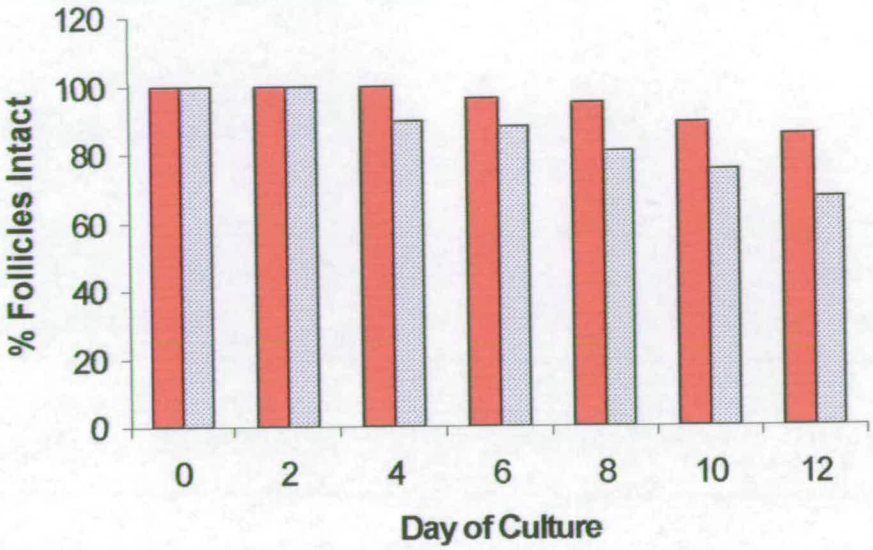


Figure 5.2. (a) Effect of ascorbic acid on follicular growth. Ascorbic acid (squares, n=82), Control (circles, n=81).
(b) Effect of serum on follicular growth. FBS added Day 0 (closed circles, n=20), FBS added Day 6 (squares, n=33), Ascorbic acid with FBS added Day 0 (open circles, n=20), Ascorbic acid with FBS added Day 6 (triangles, n=33). Values are mean ± SEM. n represents the number of follicles in each treatment group at the beginning of culture.

5.3.2. Follicular Integrity

There was a negative correlation ($p < 0.05$) between percentage follicle growth (as described above) and the percentage of follicles remaining intact during the 12 day culture period. For follicles cultured for 10 days, there was a significant correlation only in the absence of ascorbic acid ($p < 0.05$). As illustrated in Figure 5.3, from Day 8 of culture onwards, significantly ($p < 0.05$) more ascorbic acid-treated follicles remained intact in the absence of serum compared to control follicles and follicles treated with serum from Day 0. After 10 days, follicles treated with serum from Day 0 had a significantly greater loss of basement membrane integrity than follicles in all other treatment groups ($p < 0.05$). By Day 12, a higher proportion of follicles had remained intact in the serum-free ascorbic acid-treated group than in any other group ($p < 0.05$).

(a)



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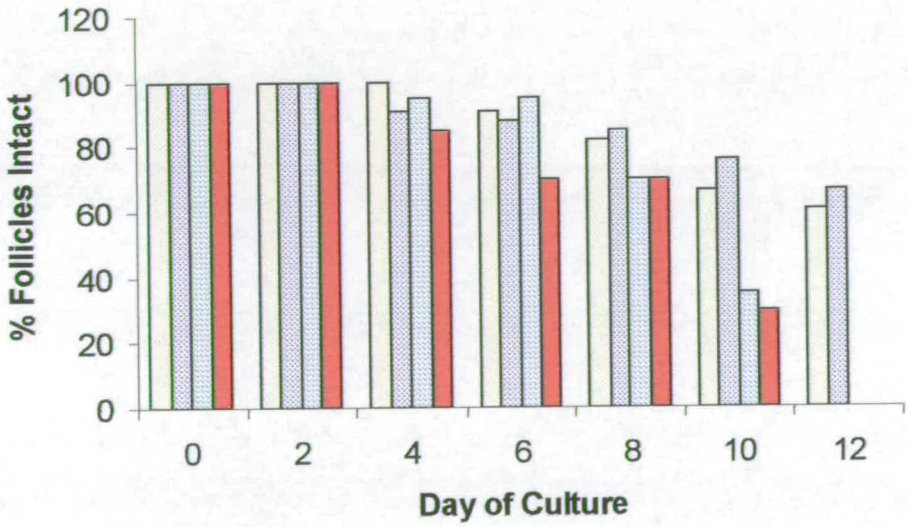


Figure 5.3. (a) Effect of ascorbic acid on follicular integrity. Ascorbic acid (red bars, n=82), Control (blue bars, n=81).
(b) Effect of serum on follicular integrity. Ascorbic acid with FBS added Day 6 (yellow bars, n=33), FBS added Day 6 (blue bars, n=33), Ascorbic acid with FBS added Day 0 (green bars, n=20), FBS added Day 0 (red bars, n=20). n represents the number of follicles in each treatment group at the beginning of culture.

5.3.3. Histological Assessment

Histological observations were made on control follicles on Day 6 (n=22) and on ascorbic acid-treated follicles in serum-free medium on Day 6 (n=10) and Day 12 (n=11) of culture. Granulosa cell degeneration (expressed as % of follicles with >5% pyknotic nuclei) was greater in control follicles than in ascorbic acid-treated follicles on Day 6 of culture ($p < 0.05$) (Table 5.1). However, the number of degenerate oocytes was significantly higher on Day 12 than on Day 6 in ascorbic acid-treated follicles ($p < 0.001$) (Table 5.2). Examples of a follicle with healthy and degenerate oocytes are shown in Figure 5.4.

Table 5.1. Effect of ascorbic acid on the percentage of follicles with pyknotic granulosa on Day 6 of culture

Treatment	Granulosa Degeneration (% Follicles)
Control[a]	18.2
Ascorbic Acid[b]	0[c]

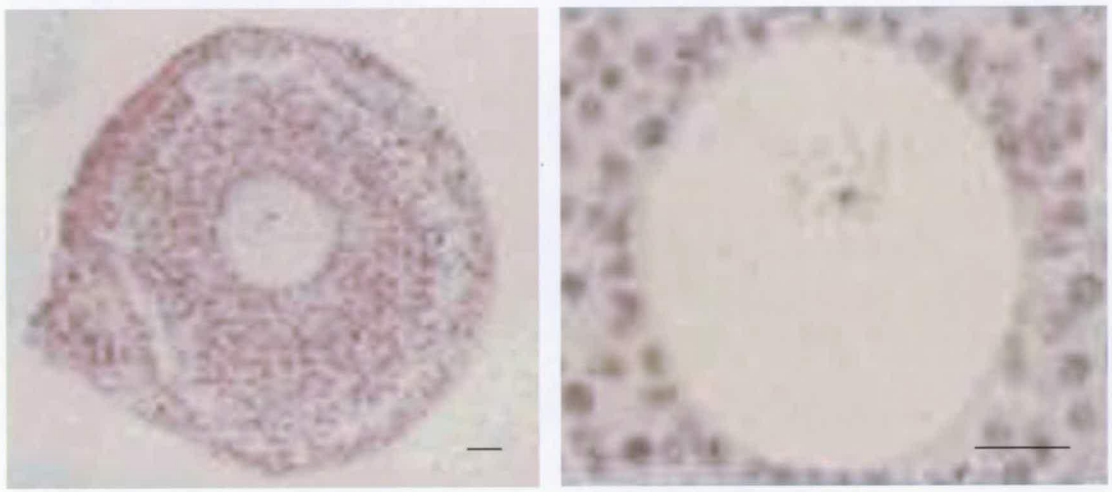
[a]n=22, [b]n=10, [c]Significantly lower than control ($p < 0.05$).

Table 5.2. Oocyte health on Day 6 and Day 12 of culture in the presence of ascorbic acid

Treatment	Oocyte Degeneration (% Follicles)
Day 6[a]	30
Day 12[b]	90.9[c]

[a]n=10, [b]n=11, [c]Significantly higher than Day 6 ($p < 0.001$).

(a) (i) (ii)



(b)

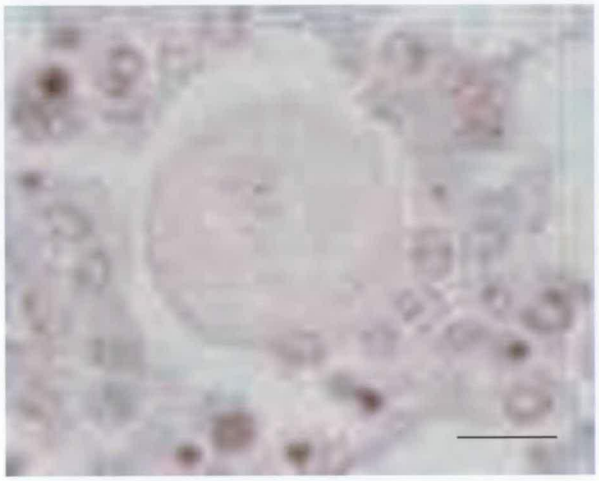


Figure 5.4. Histological sections representing healthy and degenerating oocytes. (**a**) (i) a healthy follicle with (ii) a healthy oocyte, **(b)** an unhealthy oocyte showing detachment from the granulosa cells. Bar = 20 μm.

5.3.4. Detection of Apoptosis by TUNEL

Freshly isolated follicles (Day 0; n=8), and follicles cultured for 12 days (control, n=9; ascorbic acid, n=9; serum added Day 6, n=8; ascorbic acid with serum added Day 6, n=9) were stained for the occurrence of cell death by TUNEL. The follicle sections containing the oocyte germinal vesicle were analysed using confocal microscopy and image analysis. The occurrence of cell death in the granulosa compartment was significantly different between all treatment groups ($p< 0.05$) (Table 5.3). There was no granulosa cell death in freshly isolated follicles or those cultured with ascorbic acid (with or without serum). There was significantly ($p< 0.001$) more granulosa cell death in control follicles and serum-treated control follicles than in day zero follicles or in follicles treated with ascorbic acid alone. Follicles treated with serum had healthier granulosa cells when ascorbic acid was also present ($p< 0.001$). In addition, follicles cultured with ascorbic acid displayed a significantly ($p< 0.01$) lower incidence of theca cell death than controls (Figure 5.5). Examples of a follicle with healthy granulosa cells and a follicle undergoing granulosa cell death are shown in Figure 5.6.

Table 5.3. Effect of ascorbic acid and serum on granulosa cell apoptosis on Day 12 of culture

Treatment	Apoptotic Granulosa Cells (mean % ± SEM)[a]
Day 0	0 ± 0
Control (Day 12)	6.44 ± 3.23[b]
Ascorbic Acid (Day 12)	0 ± 0
FBS (Day 12)	12.43 ± 4.13[b]
Ascorbic Acid + FBS (Day 12)	0 ± 0

[a]SEM = standard error of the mean, [b]significantly greater than Day 0.

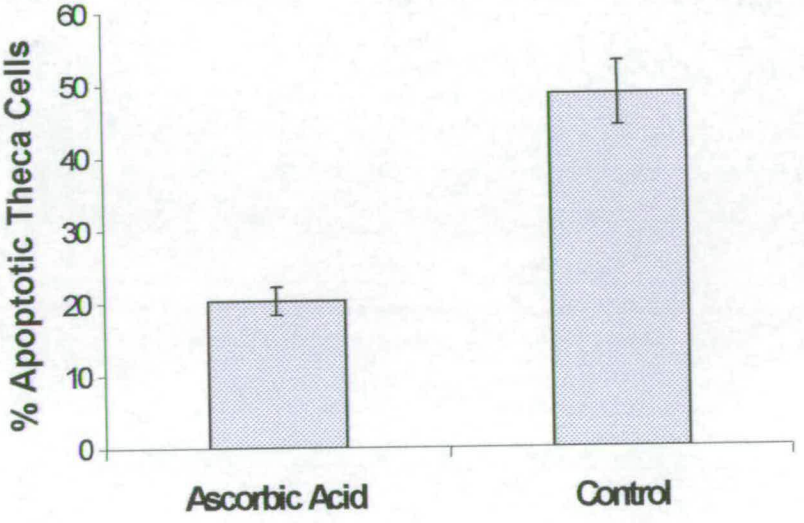


Figure 5.5. Effect of ascorbic acid on theca cell apoptosis. Expressed as fluorescein dUTP labelled cells; mean % ± SEM; n=9.

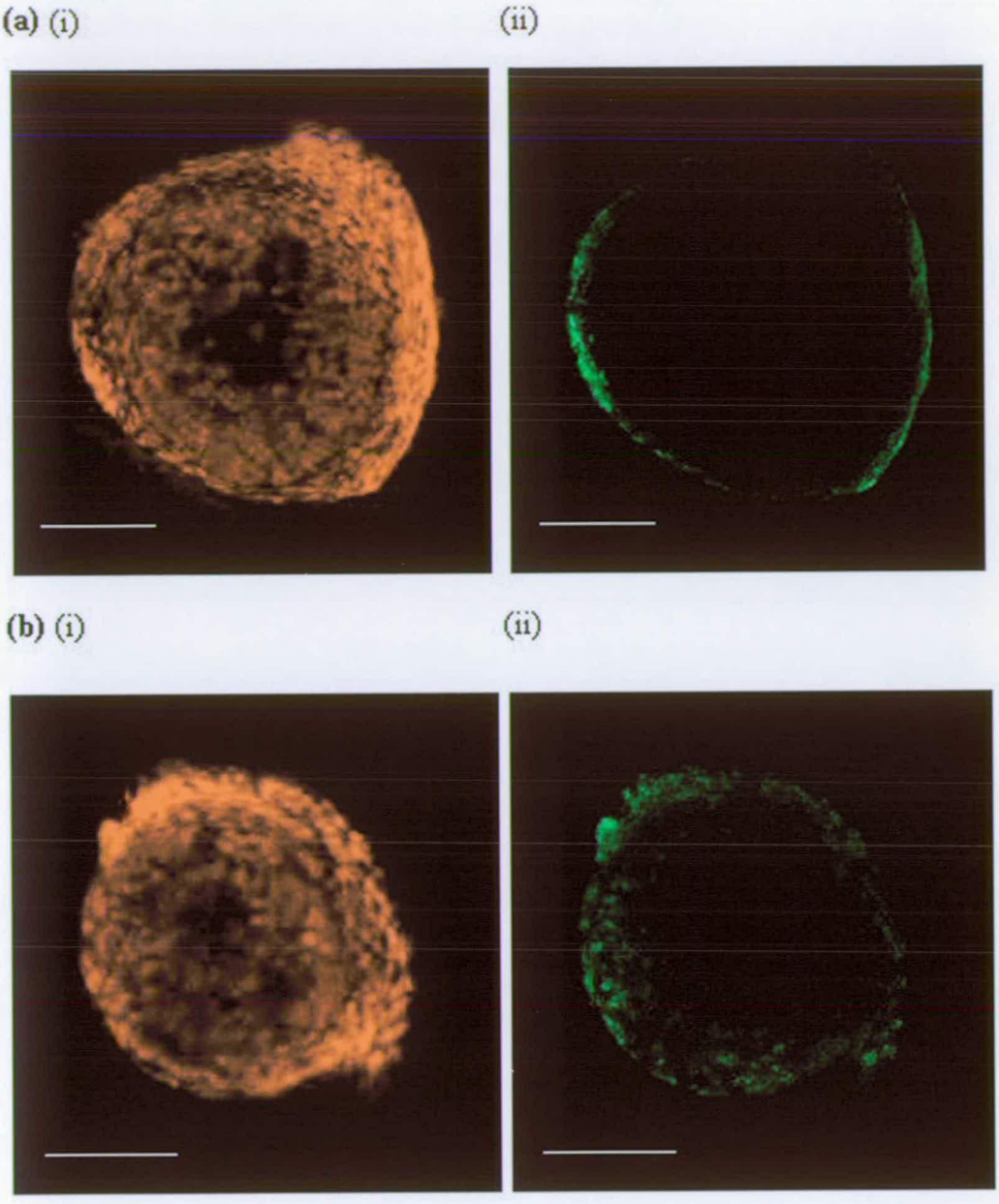


Figure 5.6. Detection of apoptosis by TUNEL
(a) ascorbic acid-treated follicle on Day 12 of culture: (i) Follicle stained with propidium iodide (red), (ii) Apoptotic theca cells labelled with fluorescein dUTP (green), no apoptotic granulosa cells present. **(b)** Control follicle on Day 12 of culture: (i) Follicle stained with propidium iodide (red), (ii) Apoptotic mural granulosa cells and theca cells labelled with fluorescein dUTP (green). Bar = 100 μm.

5.3.5. Oestradiol Secretion

During the 12 day culture period, there was significant ($p < 0.01$) oestradiol secretion by follicles in serum-free medium in the presence and absence of ascorbic acid (Figure 5.7). No significant differences in oestradiol secretion between ascorbic acid and control groups were found.

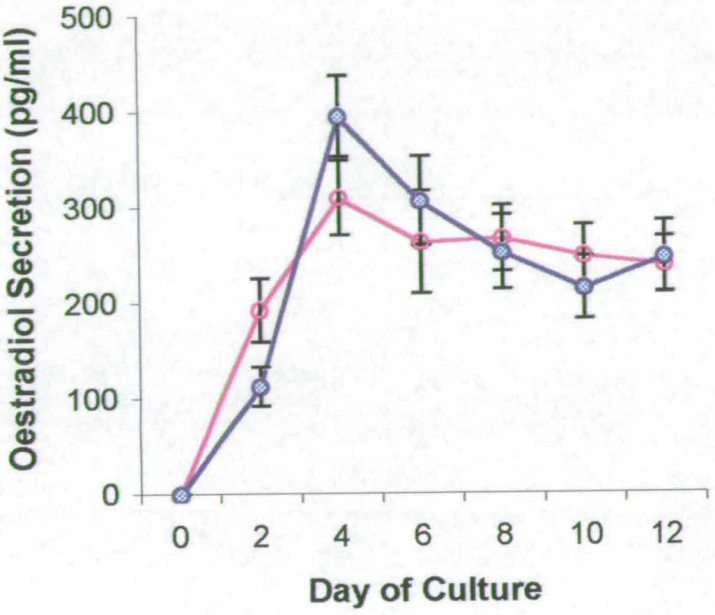


Figure 5.7. Effect of ascorbic acid on oestradiol secretion. Follicles were cultured in the presence (open circles) or absence (closed circles) of ascorbic acid. Values are mean ± SEM.

5.3.6. MMP-2 and MMP-9 Secretion

Ascorbic acid-treated (n=12) and control medium (n=12) from Day 8 of culture were analysed for MMP activity. MMP-2 (72 kDa) and MMP-9 (92 kDa) were detected in all samples. Densitometric analysis showed that latent MMP-9 activity was significantly higher in conditioned medium from ascorbic acid-treated follicles ($p < 0.05$).). The densitometric readings, taken within the range of linear sensitivity of the zymogram gel, represent a 1.5 fold increase (3748 to 5784 densitometric units) in enzyme activity. An example of a zymogram demonstrating MMP-2 and MMP-9 activity is shown in Figure 5.8.

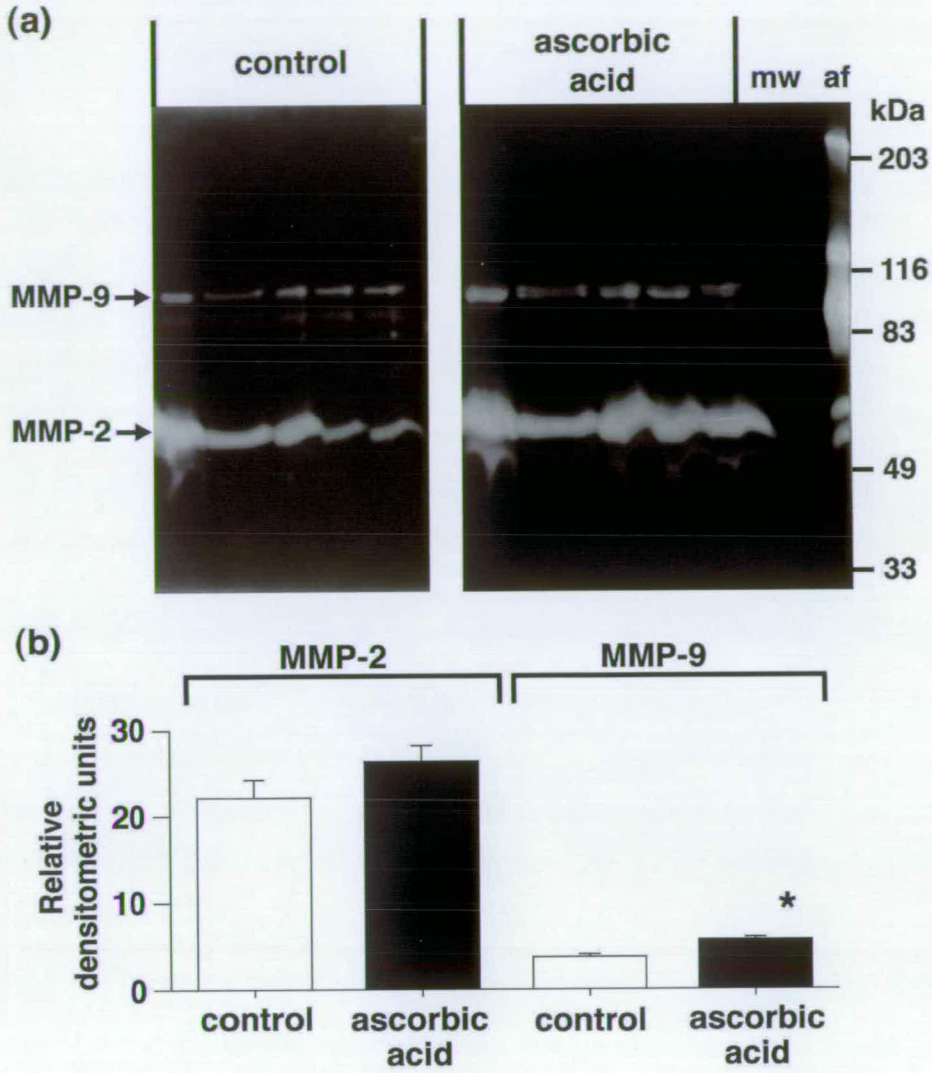


Figure 5.8. (a) Representative gelatin zymography gels showing gelatinase activities in conditioned medium collected from bovine follicles maintained in culture for eight days, either untreated (control) or treated with ascorbic acid. Gelatinase activities corresponding to the latent forms of MMP-2 and MMP-9 are indicated. A sample of human amniotic fluid collected at term was used as a positive control (af). Molecular weight markers (mw) are as indicated (kDa).
(b) Densitometric analysis (expressed as relative densitometric units; mean ± SEM; n=12) of the gelatinase activities of the latent forms of MMP-2 and MMP-9 in conditioned medium collected from bovine follicles after 8 days in culture, with no treatment (control) or treated with ascorbic acid. * significantly ($p < 0.05$) greater MMP-9 activity than controls.

5.4. DISCUSSION

The results presented here demonstrate successful growth and differentiation of bovine preantral follicles over 12 days in a serum-free culture system. We have also shown that ascorbic acid maintains follicle integrity in the absence of serum throughout the culture period, reduces the incidence of apoptotic cell death, and may participate in the regulation of remodelling of the extracellular matrix.

Ideally, a long term culture system would be serum-free in order to allow the definition of optimal conditions for follicle development. Replacement of serum with ascorbic acid in our culture system did not significantly increase follicle growth, but it did allow the follicles to maintain their morphology throughout the culture period. We have determined that increased rate of follicle growth correlates with significantly decreased follicle integrity during the 12 day culture period. The presence of serum in culture medium may have accelerated follicle development at a rate which could not be sustained, which led to follicle rupture. In addition, there was substantial theca cell loss from follicles in serum-treated groups (results not shown).

Little is known of the regulators of follicle growth during the gonadotrophin-independent stages of development. Early evidence for the role of ascorbic acid has come from the use of scorbutic guinea pigs (Kramer *et al.*, 1933), which are infertile due to ascorbic acid deficiency and demonstrate degeneration in the follicle wall consistent with loss of basement membrane integrity (Kramer *et al.*, 1933). Although little is known of the role of ascorbic acid in facilitation of follicle expansion in the growth phase, depletion of ascorbic acid is associated with structural involution in the corpus luteum in conjunction with increased activity of matrix-degrading enzymes (Endo *et al.*, 1993). Ascorbic acid, acting as an antioxidant, has been implicated in collagen biosynthesis (Pinnell, 1985), which is essential for basement membrane construction during follicle growth.

Regulated degradation of the ECM may provide an important mechanism for regulation of growth factor availability and activity during follicle development (McIntush and Smith, 1998). With respect to the preantral and early antral stages of follicular development, the contribution of the various MMPs and associated

regulators to follicular remodelling is not well understood. Work described in the previous chapter demonstrated that secretion of MMP-9 and TIMP-1 and -2 from bovine preantral follicles during culture is indicative of follicle health at 6 days. In the current study, MMP-2 and -9 are secreted by follicles on Day 8 of culture in the presence and absence of ascorbic acid. This indicates that the majority of follicles are able to proceed to this stage and remain healthy in basic serum-free culture medium. However, some control follicles were found to have pyknotic granulosa cells on Day 6 of culture, whereas in the presence of ascorbic acid, these were not detected. From Day 8 onwards there was an increased loss of basement membrane integrity in the absence of ascorbic acid, and by Day 12 there is an increase in apoptotic cell death in these groups. Our results are in agreement with other *in vitro* studies (Rose *et al.*, 1999; Murray *et al.*, 2001), which have demonstrated promotion of follicle integrity and survival by ascorbic acid in cultured mouse follicles. The study by Murray *et al.* (2001) also reported an increase in MMP and TIMP activity in the presence of ascorbic acid.

The zymography results presented here emphasise the usefulness of MMP detection as a means of monitoring follicle health throughout culture. This is illustrated by the finding that granulosa cell death (as assessed by the level of pyknosis) was detected in control follicles on Day 6 of culture, but not in the presence of ascorbic acid; and subsequently (on Day 8 of culture) the level of MMP-9 was found to be lower in medium from control follicles than in ascorbic acid-treated groups. Therefore, as ascorbic acid increased MMP-9 activity, there may be a role for ascorbic acid both in the regulation of ECM turnover and in the maintenance of follicle health during long-term culture. In the previous chapter, the importance of maintaining healthy theca cells and their connections with the granulosa layers in bovine follicle culture was emphasised, and a role for these cell types in basement membrane remodelling during the early stages of folliculogenesis was proposed. However, other studies have reported follicle rupture or loss of theca cells from bovine preantral follicles during culture (Ralph, 1996; Gutierrez *et al.*, 2000). As we have shown here that ascorbic acid significantly reduces theca cell death *in vitro*, this may help to overcome these problems and facilitate normal folliculogenesis *in vitro*.

In order to maintain health and normal development of the oocyte *in vitro*, it is important to define conditions which are conducive to somatic cell survival. The role of ascorbic acid in the neutralisation of free radical species has been well studied. As well as having relevance to follicle remodelling, this action of ascorbic acid affects the process of follicular cell death. Tilly and Tilly (1995) reported that accumulation of free radical species in tropic-factor deprived follicles is involved in triggering granulosa cell apoptosis. In that study, FSH suppressed apoptosis in cultured rat antral follicles, and this effect was mimicked by antioxidants including ascorbic acid. In our culture system, follicles treated with ascorbic acid had a significantly lower incidence of apoptotic cell death in granulosa and theca cells compared to control follicles in serum-free medium. However, the histological data shows that oocyte health declined between Day 6 and 12 of culture. This indicates that although the culture conditions are sufficient for maintenance of granulosa cell health, more research is required to identify factors which will be beneficial to oocyte development *in vitro*. Interestingly, when serum was added to follicles on Day 6 of culture, follicles had significantly more apoptotic granulosa cells by Day 12 than controls or ascorbic acid-treated follicles. However, this effect was not apparent when serum was added in conjunction with ascorbic acid. As more follicles were found to lose basement membrane integrity when serum was present, any deleterious effects may be due to inappropriate stimulation of follicles by serum during the early stages of development. Alternatively, serum factors may have a direct negative effect on immature follicles in culture.

Ascorbic acid had no significant effect on the production of oestradiol by follicles during the culture period. This finding is in agreement with a study using cultured mouse follicles (Murray *et al.*, 2001). Ascorbic acid is thought to play a similar role in steroidogenesis in the ovary as in the adrenal gland, where high concentrations inhibit steroid biosynthesis through inhibition of hydroxylation systems (Kitabchi, 1967). However, the precise mechanism through which this is effected is not well understood. Since ascorbic acid uptake in granulosa cells is positively regulated by FSH and IGF-1 in a synergistic manner similar to the control of granulosa cell differentiation (Giudice, 1992; Behrman *et al.*, 1996) ascorbic acid may

play an important part in expansion, survival and steroidogenesis in the later stages of folliculogenesis and during the establishment of dominance.

In conclusion, we have shown that ascorbic acid improves the survival of follicles in a serum-free culture system, and reduces somatic cell death. In addition, we have reported a role for ascorbic acid in the regulation of basement membrane remodelling by influencing MMP production. Ascorbic acid had no effect on oestradiol production or follicle growth in this study. As oocyte health was found to decline during the culture period, further investigation is required in order to identify factors necessary for improvement of oocyte quality *in vitro*. In chapter 6, we will discuss a putative role for IGF-1 in the regulation of oocyte development *in vitro*.

Chapter 6

Role of the IGF System in Preantral Follicle Development

6.1. INTRODUCTION

As discussed in previous chapters, IGFs are important regulators of follicular function. However, there appears to be differential regulation of these factors according to developmental stage, as well as species differences regarding spatial and temporal action.

The bioactivity of IGFs is controlled by their association with a family of specific IGF binding proteins (IGFBPs), which are found in association with the extracellular matrix (ECM) and cell membranes. In many mammalian species, follicular growth and atresia are characterised by dramatic changes in follicular IGFBP levels (e.g. sheep: Monget *et al.*, 1993; cow: Echternkamp *et al.*, 1994; pig: Grimes *et al.*, 1994; human: Cataldo and Giudice, 1992). Since IGFBPs have equal or higher affinity for IGFs than do IGF receptors (Shimasaki *et al.*, 1991), the degradation of IGFBPs by specific IGFBP-degrading proteinases may be an important step in regulating the actions of IGFs (Fowlkes *et al.*, 1995).

The IGF-1 analogue LR3 IGF-1, which does not bind to IGF binding proteins, is widely used in follicle and granulosa cell culture systems. Recently, this factor was reported to increase growth of bovine preantral follicles in a long term culture (Gutierrez *et al.*, 2000). We reported in chapter 3 that this IGF-1 analogue does not increase follicular growth during a six day culture period, and have hypothesised that the follicles may not have differentiated sufficiently *in vitro* to respond appropriately to this potent form of IGF-1. In the current chapter we will determine whether LR3 IGF-1 has any effects on the proliferation and morphology of the follicles cultured in experiments described in chapter 3. Follicles cultured in the presence of recombinant IGF-1 will also be examined using histological and immunocytochemical methods, in order to determine whether allowing IGF-1 to be sequestered by IGFBPs has any effect on the development of the oocyte and follicular cells.

6.2. MATERIALS AND METHODS

6.2.1. Follicle Isolation and Culture

Preantral follicles (100-200 μm) were dissected from bovine ovaries and cultured for 6 days as described in chapter 2.

6.2.2. Treatments

6.2.2.1. Treatment with LR3 IGF-1 in the absence of ascorbic acid

Follicles were cultured for 6 days in control medium (n=61) or in the presence of 10 ng/ml LR3 IGF-1 (n= 72), as described in 3.2.2.1(a).

6.2.2.2. Treatment with recombinant IGF-1 and LR3 IGF-1 in the presence of ascorbic acid

Follicles were cultured in control medium (n=79) or in the presence of 10 ng/ml recombinant IGF-1 (n=80) or LR3 IGF-1 (n=79), as described in 3.2.2.3.

6.2.3. Histological Assessment

Follicles were processed for histological analysis as described in chapter 2.

6.2.4. Detection of Granulosa Cell Proliferation by Immunocytochemistry

At the end of the culture period, follicles were fixed in 4% paraformaldehyde, processed and mounted as described in 2.3.3. Sections (Control: n=10, LR3 IGF-1: n=12, hrIGF-1: n=13) were assessed for cell proliferation using a Ki67 antigen detection kit as described in 2.3.3.3.

6.2.5. Statistical Analyses

Oocyte diameters from freshly isolated and cultured preantral follicles were compared using a one-way ANOVA, with subsequent t-tests to allow individual comparisons between groups. Oocyte diameters are also represented graphically using box and whisker plots. In experiment 6.2.2.1, the number of follicular granulosa layers present at the end of culture was compared between groups using a Chi-square test. In experiment 6.2.2.2, the mean percentage of proliferating cells on Day 6 of culture was compared using a one-way ANOVA, followed by 2-sample *t*-tests.

6.3. RESULTS

6.3.1. Histological Assessment

6.3.1.1. Effect of LR3 IGF-1 in the absence of ascorbic acid

Histological observations were made using freshly isolated follicles (n=10), LR3 IGF-1 treated follicles (n=24) and control follicles (n=22). A significant difference in oocyte diameter between the three groups was detected by ANOVA ($p< 0.01$), and follicles treated with LR3 IGF-1 had significantly smaller oocytes than controls and freshly isolated follicles ($p< 0.01$) (Figure 6.1). Follicles cultured with LR3 IGF-1 also had a reduced number of granulosa cell layers ($p< 0.01$) by day 6 of culture (Table 6.1). However, LR3 IGF-1 had no effect on the percentage of follicles with degenerating oocytes and granulosa cells, the thickness and level of pyknosis of the follicular theca layers, nor on the percentage of follicles which formed an early antrum *in vitro* (Table 6.1). Examples of follicles with healthy and degenerating somatic cells are shown in Figure 4.3.

Table 6.1. Effect of LR3 IGF-1 on histological measurements on Day 6 of culture

Treatment	Mean Oocyte Diameter (μm ± SEM)[a]	Mean No. Granulosa Layers (± SEM)	Mean No. Theca Layers (± SEM)	Oocyte Degeneration (% Follicles)	Granulosa Degeneration (% Follicles)	Theca Degeneration (% Follicles)	Antrum Formation (% Follicles)
Control[b]	52.4 ± 3.60	9.3 ± 0.41	3.9 ± 0.53	18.2	18.2	47.4	40.1
LR3 IGF-1[c]	37.8 ± 2.34[d]	6.4 ± 0.30[d]	3.0 ± 0.44	37.5	16.7	58.8	33.3

[a] SEM = standard error of the mean.
[b] n = 22
[c] n = 24
[d] Significantly lower than control ($p < 0.01$).

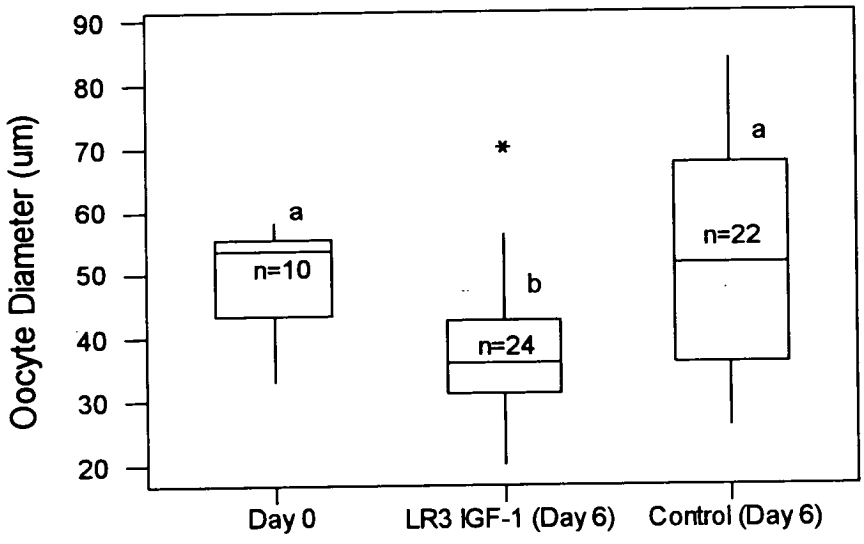


Figure 6.1. Effect of LR3 IGF-1 on oocyte diameter. Box and whisker plot of histological measurements of mean oocyte diameter on Day 0 and Day 6 of culture. n represents the total number of follicles analysed for each treatment. Box represents the median and the upper and lower quartiles. Whiskers illustrate the expected data range (* denotes outlier). Different letters indicate significant differences ($p < 0.01$).

6.3.1.2. Effect of recombinant IGF-1 and LR3 IGF-1 on oocyte diameter

Histological observations were made using freshly isolated follicles (n=16), LR3 IGF-1 treated follicles (n=17), recombinant IGF-1 treated follicles (n=16) and control follicles (n=18) on day 6 of culture. Oocyte diameters are represented in Figure 6.2. A significant difference in oocyte diameter between the three groups was detected by ANOVA ($p< 0.05$). Only follicles treated with recombinant IGF-1 had significant oocyte growth compared to day zero oocytes. ($p< 0.01$). In addition, follicles treated with LR3 IGF-1 had significantly smaller oocytes than recombinant IGF-1 treated follicles on day 6 of culture ($p< 0.05$).

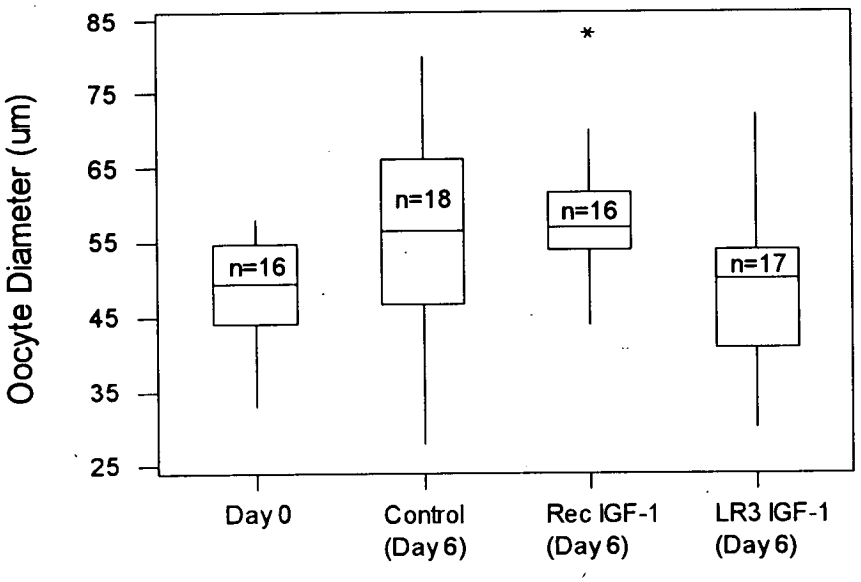


Figure 6.2. Effect of LR3 IGF-1 and recombinant IGF-1 on oocyte diameter. Box and whisker plot of histological measurements of mean oocyte diameter on Days 0 and 6 of culture. n represents the total number of follicles analysed for each treatment. Box represents the median and the upper and lower quartiles. Whiskers illustrate the expected data range (* denotes outlier). Oocyte growth is significant from day 0 to day 6 only in the presence of recombinant IGF-1 (Rec IGF-1) ($p< 0.01$).

6.3.2. Detection of Granulosa Cell Proliferation

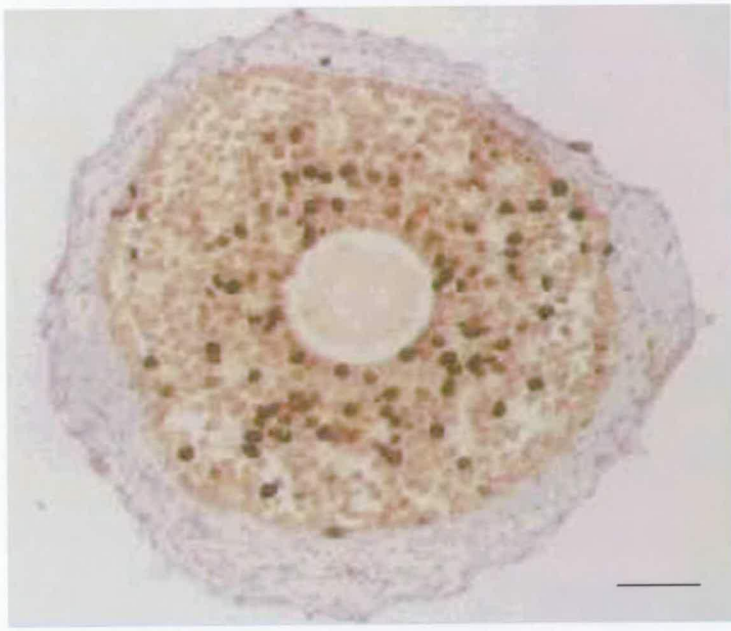
As illustrated in Table 6.2, there was a significant difference in the percentage of Ki67-positive granulosa cells between groups on Day 6 of culture ($p < 0.05$). There was significantly more proliferation in LR3 IGF-1 follicles (n=12) and recombinant IGF-1 (n=13) follicles than in controls (n=10) ($p < 0.05$). There was no difference in the percentage of proliferating cells between the two IGF-1 groups. A follicle displaying Ki67-positive staining is displayed in Figure 6.3.

Table 6.2. Effect of IGF-1 on granulosa cell proliferation on Day 6 of culture

Treatment	Proliferating Granulosa Cells (mean % ± SEM)
Control	8.04 ± 2.45
Recombinant IGF-1	16.63 ± 2.82[a]
LR3 IGF-1	18.43 ± 3.16[a]

[a] Significantly higher than control ($p < 0.05$).

(a)



(b)

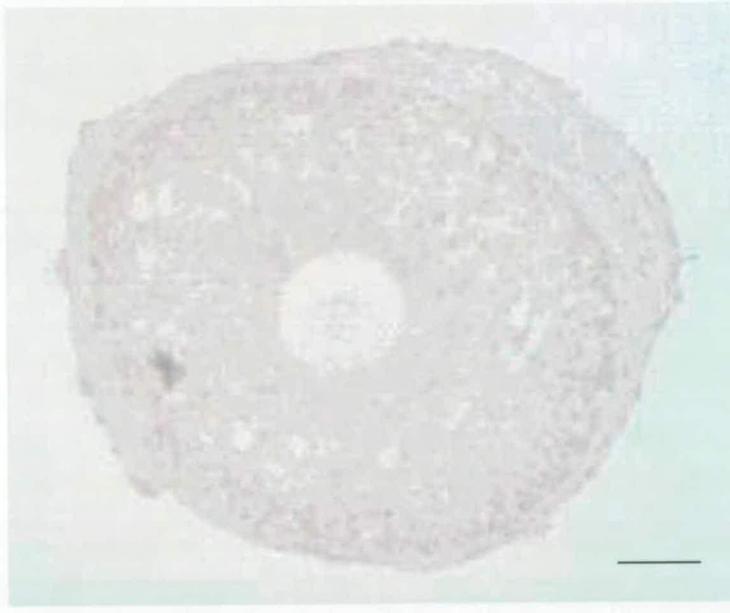


Figure 6.3. Detection of proliferation by Ki67 immunostaining. **(a)** Follicle showing Ki67-positive staining (dark brown) in granulosa cells. **(b)** Negative control follicle. Bar = 50 μm.

6.4. DISCUSSION

The results presented here demonstrate a significant effect of recombinant IGF-1 and the analogue LR3 IGF-1 on follicle proliferation in bovine preantral follicles cultured for 6 days in a serum-free system. In the initial experiment, we demonstrated that LR3 IGF-1 exerts a negative effect on oocyte development and the number of granulosa cell layers during preantral and early antral stages of folliculogenesis. Subsequently, we confirmed that LR3 IGF-1 adversely affects oocyte growth *in vitro*. In addition, we determined that recombinant IGF-1, which associates with IGFBPs, does not produce this deleterious effect.

Within bovine antral follicles, there is evidence of a complete intrafollicular IGF system (Armstrong *et al.*, 1998, 2000) in which follicles express IGF ligand, and mediation of IGF action and bioavailability occurs through receptors and binding proteins. The expression of mRNA encoding IGF-1 is developmentally regulated in a species-specific manner (Armstrong and Webb, 1997; Webb and Armstrong, 1998).

As discussed in chapter 3, immunoreactive type 1 IGF receptor has been visualised in oocytes and granulosa cells of primordial, preantral and antral human follicles (Qu *et al.*, 2000) which implies a role for IGFs during early follicle and oocyte development. We have shown here that recombinant IGF-1 and LR3 IGF-1 stimulate proliferation of preantral follicles. However, the precise role of IGFs in regulation of oocyte development during early folliculogenesis is unclear. IGF-1 stimulates nuclear maturation of cumulus-enclosed oocytes (Gomez *et al.*, 1993; Yoshimura *et al.*, 1996; Pawshe *et al.*, 1998), and improves early embryonic development (Pawshe *et al.*, 1998). Moreover, IGF-1 knockout mice have reduced glucose transporter expression, which is thought to be essential for oocyte maturation and successful ovulation (Zhou *et al.*, 2000). Since 'maternal' factors which accumulate in the oocyte may have a long lasting effect on subsequent development (Revel *et al.*, 1995), IGF-1 may exert an effect on oocyte maturation in early follicle stages by binding to the cell surface receptors long before oocyte competence has been achieved.

We have demonstrated here that treatment of immature follicles with LR3 IGF-1 adversely affects oocyte growth. *In vivo*, IGFBPs control IGF bioactivity by regulating its availability to IGF receptors (Jones and Clemmons, 1995). For example, IGFBP-1 has been shown to inhibit the synergistic effect of IGF-1 and FSH in stimulating progesterone production by cultured rat granulosa cells (Bicsak *et al.*, 1990). In addition, IGFBP-3, which is expressed in bovine theca cells (Webb *et al.*, 1999), has been shown to inhibit the ability of IGF-1 to stimulate proliferation, progesterone and androstenedione production by bovine theca cells (Spicer *et al.*, 1997). Furthermore, during bovine follicular growth and atresia, there are distinct spatial and temporal changes in the expression of IGFBP-2 and –4 mRNA *in vivo* (Armstrong *et al.*, 1998).

Interestingly, the presence of the Booroola (Fecundity) gene in sheep, characterised by increased ovulation rate (Davis *et al.*, 1982) and precocious follicular maturation (Henderson *et al.*, 1985; McNatty *et al.*, 1986), correlates with reduced intrafollicular IGFBP concentrations compared with follicles from sheep which do not carry the mutation (Monniaux *et al.*, 2000). The increased availability of IGF at an earlier stage may, at least in part, have an effect on the acquisition of oocyte meiotic competence thus leading to increased ovulation rate in these animals. During early antral development in bovine follicles, IGFBP-2 from the granulosa cells may regulate the bioavailability of IGFs (Armstrong *et al.*, 1998). At the appropriate stage of development, specific IGFBP proteinases release bound IGFs, thus allowing IGF to act upon on the follicle or oocyte (Logan and Hill, 1992). The origin and regulation of intrafollicular proteinase(s) degrading IGFBPs in the ovary are still unclear. Calcium and zinc-dependent metalloproteinases may be involved in porcine intrafollicular IGFBP proteolytic activity (Besnard *et al.*, 1997). However, experiments using a synthetic MMP inhibitor determined that 60, 70 and 72 kDa MMPs are not of primary importance in the degradation of intrafollicular IGFBPs < 40 kDa (IGFBP-2, -4 and –5) in the pig (Besnard *et al.*, 1997).

As LR3 IGF-1 does not bind to IGFBPs, we have previously suggested that over-stimulation of immature follicles by this IGF-1 analogue may lead to precocious differentiation which impedes oocyte development. In the current chapter we have

shown that regulation of the bioavailability of IGF-1 by IGFBPs promotes oocyte growth *in vitro*. If MMPs are involved in facilitation of this action of IGF-1 on the oocyte, these enzymes may also be useful markers for oocyte development *in vitro*.

In conclusion, we have investigated the effects of IGF-1 on bovine preantral follicle development in a serum-free culture system, and shown that recombinant IGF-1 and the analogue LR3 IGF-1 stimulated follicle proliferation during a six day culture period. However, LR3 IGF-1 had a damaging effect on oocyte development at the preantral and early antral stages, whereas recombinant IGF-1 promoted oocyte growth *in vitro*. These experiments highlight the importance of the IGF system in the early stages of follicle development, and emphasise the need for preserving physiological control mechanisms during follicle culture. These results also suggest that it may be necessary to dissociate oocyte and somatic cell requirements in order to allow normal oocyte development to occur *in vitro*.

Chapter 7

General Discussion

7.1. General Discussion

An abundant and homogeneous source of oocytes for use in *In vitro* maturation (IVM) systems could be obtained by growing oocytes from preantral follicles *in vitro*. However, culture systems for bovine and ovine follicles have not been successful in terms of producing meiotically competent oocytes (reviews: Telfer, 1998b; Telfer *et al.*, 2000). In order to advance this technology, recent culture systems have concentrated on defining conditions for the early stages of follicle development (Figueiredo *et al.*, 1994; Hulshof *et al.*, 1995; Cecconi *et al.*, 1999).

The aims of this thesis were (I) to investigate factors affecting early bovine follicular development using a defined culture system, and (II) to identify markers of early follicular development *in vitro*. The main findings from this study, alongside information from other researchers, are illustrated in Figure 7.1. The identified factors may, at specific stages of development, play a significant role in bovine follicle and oocyte development *in vitro*.

Ovarian folliculogenesis is regulated by both endocrine and intraovarian mechanisms which co-ordinate the processes of somatic cell proliferation and differentiation. As discussed in chapter 1, regulation occurs at various levels, with a constantly shifting contribution by endocrine, autocrine and paracrine factors. Furthermore, the oocyte may participate in the control of its own development as well as that of the follicular cells. Evidence from studies in domestic ruminants shows that locally produced growth factors together with receptors for both growth factors and gonadotrophins are expressed during early follicle development in a stage- and cell-specific manner (review: McNatty *et al.*, 1999). In chapter 3, preliminary experiments determined that bovine preantral follicles are capable of growth and differentiation (as measured by oestradiol secretion and antrum formation) during a six day serum-free culture period. FSH and IGF-1 did not significantly increase follicular diameter, although FSH was found to stimulate follicular oestradiol secretion. Hence FSH is probably affecting granulosa cell differentiation in this system. The dissociation of follicular growth from steroidogenenic function indicates that measurement of follicular diameter may not be a reliable marker of physiological follicular

development *in vitro*. Subsequent chapters proceeded to further investigate follicle and oocyte development during the early stages of folliculogenesis. Furthermore, rather than relying on follicle growth as a indicator of follicle development, follicle and oocyte morphology were examined *in vitro*.

It is becoming increasingly clear that the follicular ECM plays an important part in many of the regulatory processes during folliculogenesis. For example, numerous *in vitro* studies have shown that cell morphology is altered according to the type of ECM component on which the cells are cultured (Watt, 1986). In addition, the follicular basal lamina is believed to play a role in influencing granulosa cell proliferation and differentiation (Amsterdam *et al.*, 1989; Richardson *et al.*, 1992; Luck, 1994). During follicular development, turnover and reconstruction of the follicular ECM is facilitated and regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). In chapter 4, secretion of MMPs and TIMPs from preantral follicles during a six day culture period was measured. It was demonstrated that when MMP-9 and TIMPs are secreted *in vitro*, a follicle has a higher probability of being healthy at the end of the culture period. If oocyte development is to occur in species with a long growth period, an extended culture period will be necessary. Therefore, non-invasive markers of follicular development are required. Since MMPs and TIMPs can be detected in conditioned culture medium, these factors are candidates for this role.

The experiments described in chapter 3 and chapter 6 determined that IGF-1 increased granulosa cell proliferation in preantral follicles during a six day culture. However, LR3 IGF-1, an analogue which does not bind to IGF binding proteins, was found to have a detrimental effect on oocyte development, whereas recombinant IGF-1 promoted oocyte growth *in vitro*. These experiments highlight the importance of the IGF system in the early stages of oocyte development, and emphasise the need for preserving physiological control mechanisms during follicle culture.

Growth factors influence follicular development, in part, by stimulating DNA synthesis and by modulating the onset of apoptosis in granulosa cells. For example, both IGF-1 and EGF exert inhibitory influences on apoptosis in various cell types including granulosa cells (Tilly *et al.*, 1992; Luciano *et al.*, 1994). IGF binding

proteins are important components of the ECM that can have a major influence on cellular responses to IGF-1 (review: Giudice, 1992). Proteolysis of the ECM is a key process in regulation of availability of IGF-1 activity within developing follicles (Figure 7.1). MMPs have been reported to function as IGFBP-degrading proteinases, both *in vitro* and *in vivo* (Fowlkes *et al.*, 1994a; Fowlkes *et al.*, 1994b; Thrailkill *et al.*, 1995). However, within the ovary, the origin and regulation of the IGFBP-specific proteinase(s) is unknown. A previous study in the rat indicated that the proteinase(s) may partly derive from granulosa cells (Liu *et al.*, 1993). In contrast, IGFBP-specific proteolytic activity is produced by the theca and stroma of human ovaries (Mason *et al.*, 1996). It is becoming increasingly apparent that the oocyte produces factors that affect follicular development and function (Dong *et al.*, 1996). Therefore, a role for the oocyte in regulation of IGF action requires investigation. Furthermore, as we have already made some progress in identifying secreted markers of follicle health *in vitro*, identification of specific proteinase(s) which degrade IGFBPs should significantly advance our ability to monitor oocyte progress during long term culture.

In order to maintain health and normal development of the oocyte *in vitro*, it is important to define conditions which are conducive to somatic cell survival. Ascorbic acid has been implicated in several processes associated with follicular development, including collagen biosynthesis, steroidogenesis and apoptosis (review: Luck *et al.*, 1995). The role of ascorbic acid in the neutralisation of free radical species has been well studied. As well as having relevance to follicular remodelling, this action of ascorbic acid affects the process of follicular cell death. It was determined in chapter 5 that a higher proportion of follicles remained intact in the presence of ascorbic acid in serum-free conditions, with significantly less granulosa and theca cell death than control follicles. Moreover, ascorbic acid significantly increased production of MMP-9, an enzyme involved in basement membrane remodelling and a marker for follicular health. Therefore, ascorbic acid may be a key factor for the maintenance of bovine follicular health and remodelling *in vitro*. It has also been shown that IGF-1 increases the uptake of ascorbic acid into granulosa cells *in vitro* (Giudice, 1992; Behrman *et al.* 1996) (Figure 7.1), thus this factor may be involved in the process of IGF-1-stimulated follicular proliferation and oocyte development.

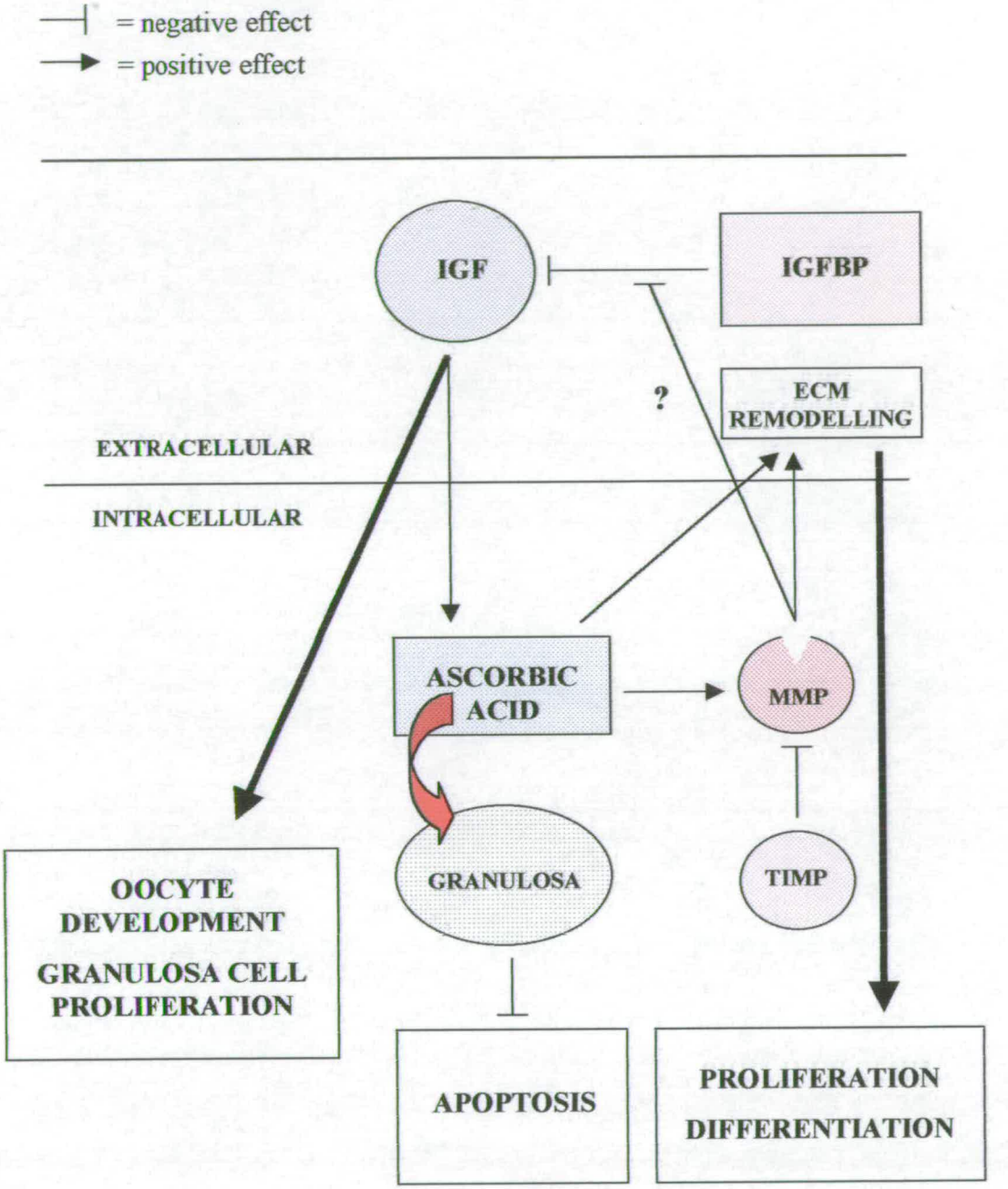


Figure 7.1. Summary of the putative developmentally-regulated factors involved in early bovine folliculogenesis.

7.2. Concluding Remarks

The aim of this thesis was to use a serum-free culture system to identify regulators of early bovine follicle and oocyte development. As well as adding to our understanding of the co-ordination of oogenesis and folliculogenesis, this research has led to the improvement of culture conditions for bovine preantral follicles and has facilitated the identification of markers of follicular health *in vitro*. The ultimate measure of the success of the culture system will be to determine whether oocytes grown *in vitro* are capable of meiotic maturation and completion of developmental competence. A technique for obtaining a source of homogeneous mature oocytes from bovine ovaries would provide a model for *in vitro* maturation (IVM) and fertilisation (IVF) systems for human oocytes, as well as permitting investigations into postovulatory and embryonic development.

Improvement of existing techniques for assisted reproduction and nuclear transfer could potentially revolutionise modern medicine. However, over the last decade there has been no real progress in applying these techniques to humans and animals with follicles that undergo a long growth period. Since folliculogenesis is a chain of many complex and precisely regulated events, gaining a better understanding of this process is key to the advancement of such technology. It is important to emphasise that although follicle culture systems are important research tools for understanding basic mechanisms of follicular development, much has still to be learned before this technology can be applied in a clinical or commercial setting.

References

Abir R, Franks S, Mobberley MA, Moore PA, Margara RA, Winston RM. Mechanical isolation and *in vitro* growth of preantral and small antral human follicles. Fertil Steril 1997; 68: 682-688

Adams CP, Evans ACO, Rawlings NC. Follicular waves and circulating gonadotrophins in 8-month-old prepubertal heifers. J Reprod Fertil 1994; 100: 27-33

Adashi EY, Resnick CE, Payne DW, Rosenfeld RG, Matsumoto T, Hunter MK, Gargosky SE, Zhou J, Bondy CA. The mouse intraovarian insulin-like growth factor system: Departures from the rat paradigm. Endocrinol 1997; 138: 3881-3890

Adashi EY, Roban RM. Intraovarian regulation. Peptidergic signalling systems. Trends in Endocrinol Metab 1992; 3: 243-248

Amsterdam A, Rotmensch S, Furman A, Venter EA, Vlodavsky I. Synergistic effect of human chorionic gonadotrophin and extracellular matrix on *in vitro* differentaition of human granulosa cells: progesterone production and gap junction formation. Endocrinol 1989; 124: 1956-1964

Armstrong DG, Baxter G, Gutierrez CG, Hogg CO, Glazyrin AL, Campbell BK, Bramley TA, Webb R. Insulin-like growth factor binding protein-2 and –4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status. Endocrinol 1998; 139: 2146-2154

Armstrong DG, Gutierrez CG, Baxter G, Glazyrin AL, Mann GE, Woad KJ, Hogg CO, Webb R. Expression of mRNA encoding IGF-I, IGF-II and type I IGF receptor in bovine ovarian follicles. J Endocrinol 2000; 165: 101-113

Armstrong DG, Webb R. Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins. Rev Reprod 1997; 2: 139-146

Armstrong DT, Goff AK, Dorrington JH. Regulation of follicular estrogen synthesis. In Midgley AR, Sadler WA (eds), Ovarian follicular development and function. New York Raven Press; 1979: 169-182

Austin EJ, Mihm M, Evans ACO, Knight PG, Ireland JLH, Roche JF. Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. Biol Reprod 2001; 64: 839-848

Bagavandoss P. Differential distribution of gelatinases and tissue inhibitor of metalloproteinase-1 in the rat ovary. J Endocrinol 1998; 158: 221-228

Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellve AR, Efstratiadis A. Effects of an IGF-1 gene null mutation on mouse reproduction. Mol Endocrinol 1996; 10: 903-918

Behrman HR, Preston SL, Aten RF, Rinaudo P, Zreik TG. Hormone induction of ascorbic acid transport in immature granulosa cells. Endocrinol 1996; 10: 4316-4321

Bergh C, Olsson JH, Hillensjo T. Effect of insulin-like growth factor I on steroidogenesis in cultured human granulosa cells. Acta Endocrinol (Copenh) 1991; 125: 177-185

Besnard N, Pisselet C, Zapf J, Hornebeck W, Monniaux D, Monget P. Proteolytic activity is involved in changes in intrafollicular insulin-like growth factor-binding protein levels during growth and atresia of ovine ovarian follicles. Endocrinol 1997; 137: 1599-1607

Bicsak TA, Shimonaka M, Malkowski M, Ling N. Insulin-like growth factor-binding protein (IGF-BP) inhibition of granulosa cell function: Effect on cyclic adenosine 3', 5'-monophosphate, deoxyribonucleic acid synthesis, and comparison with the effect of an IGF-1 antibody. Endocrinol 1990; 126: 2184-2189

Bielski BHJ, Richter HW, Chan PC. Some properties of the ascorbate free radical. Ann NY Acad Sci 1975; 258: 231-238

Billig H, Furata I, Hsueh AJW. Gonadotrophin releasing hormone (GnRH) directly induces apoptotic cell death in the rat ovary: biochemical and in situ detection of DNA fragments in granulosa cells. Endocrinol 1994; 134: 245-252

Billig H, Furuta I, Hsueh AJW. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinol 1993; 133: 2204-2212

Bodensteiner KJ, Clay CM, Moeller CL, Sawyer HR. Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. Biol Reprod 1999; 60: 381-386

Boland NI, Gosden RG. Effects of epidermal growth factor on the growth and differentiation of cultured mouse ovarian follicles. J Reprod Fertil 1994; 101: 369-374

Boland NI, Humpherson PG, Leese HJ, Gosden RG. Pattern of lactate production and steroidogenesis during growth and maturation of mouse ovarian follicles *in vitro*. Biol Reprod 1993; 48: 798-806

Boujrad N, Ogwuegbu SO, Garnier M, Lee C-H, Martin BM, Papadopoulos V. Identification of a stimulator of steroid hormone synthesis isolated from testis. Science 1995; 268: 1609-1612

Buccione R, Schroeder AC, Eppig JJ. Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biol Reprod 1990; 43: 543-547

Byrd JA, Pardue SL, Hargis BM. Effect of ascorbate on luteinizing hormone stimulated progesterone biosynthesis in chicken granulosa cells *in vitro*. Comparative Biochem and Physiol 1993; 104: 279-281

Byskov AGS. Cell kinetic studies of follicular atresia in the mouse ovary. J Reprod Fertil 1974; 37: 277-285

Cara JF, Rosenfield RL. Insulin-like growth factor I and insulin potentiate luteinising hormone-induced androgen biosynthesis by rat ovarian theca-interstitial cells. Endocrinol 1988; 123: 733-739

Cataldo NA, Giudice LC. Insulin-like growth factor binding protein profiles in human ovarian follicular fluid correlate with follicular functional status. J Clin Endocrinol Metab 1992; 74: 821-829

Cecconi S, Barboni B, Coccia M, Mattioli M. *In vitro* development of sheep preantral follicles. Biol Reprod 1999; 60: 594-601

Chaffin CL, Stouffer RL. Expression of matrix metalloproteinases and their tissue inhibitor messenger ribonucleic acids in macaque periovulatory granulosa cells: time course and steroid regulation. Biol Reprod 1999; 61: 14-21

Chiras DD, Greenwald GS. Effects of steroids and gonadotrophins on follicular development in hypophysectomised hamster. Am J Anat 1978; 152: 307-320

Chun SY, Billig H, Tilly JL, Furuta I, Tsafriri A, Hsueh JW. Gonadotrophin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor I. Endocrinol 1994; 135: 1845-1853

Clark DE, Tisdall DJ, Fidler AE, McNatty KP. Localization of mRNA encoding c-kit during the initiation of folliculogenesis in ovine fetal ovaries. J Reprod Fertil 1996; 106: 329-335

Clarke GM, Cooke D. A basic course in statistics. Edward Arnold, London; 1992: 92

Cortvrindt R, Smitz J, Van Steirteghem AC. Assessment of the need for follicle stimulating hormone in early preantral mouse follicle culture *in vitro*. Hum Reprod 1997; 12 (suppl 4): 759-768

Cortvrindt R, Smitz J, Van Steirteghem AC. *In vitro* maturation, fertilisation and embryo development of immature oocytes from early preantral follicles from prepubertal mice in a simplified culture system. Hum Reprod 1996; 11: 2656-2666

Davis GH, Dodds KG, Wheeler R, Jay NP. Evidence that an imprinted gene on the X chromosome increases ovulation rate in sheep. Biol Reprod 2001; 64: 216-221

Davis GH, McEwan JC, Fennessy PF, Dodds KG, Farquhar PA. Evidence for the presence of a major gene influencing ovulation rate on the X chromosome of sheep. Biol Reprod 1991; 44: 620-624

Davis GH, Montgomery GW, Allison AJ, Kelly RW, Bray AR. Segregation of a major gene influencing fecundity in progeny of Booroola sheep in New Zealand. NZ J Agric Res 1982; 25: 525-529

Deane HW. Histochemical observation on the ovary and oviduct of the albino rat during the estrous cycle. Am J Anat 1952; 91: 363-413

Docherty AJ, Lyons A, Smith BJ, Wright EM, Stephens PE, Harris TJR, Murphy G, Reynolds JJ. Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. Nature 1985; 318: 66-69

Dong J, Albertini DF, Nishimori K, Rajendra Kumar T, Lu N, Matzuk M. Growth differentiation factor -9 is required during early ovarian folliculogenesis. Nature 1996; 383: 531-535

Drury RAB, Wallington EA. Carleton's Histological Technique, 4th edn. Oxford University Press 1976; 126-130

Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM. The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol Endocrinol 1998; 12: 1809-1817

Dufour J, Cahill LP, Mauleon P. Short- and long-term effects of hypophysectomy and unilateral ovariectomy on ovarian follicular populations in sheep. J Reprod Fertil 1979; 57: 301-309

Duncan WC, Illingworth PJ, Fraser HM. Expression of tissue inhibitor of metalloproteinases-1 in the primate ovary during induced luteal regression. J Endocrinol 1996; 151: 203-213

Echternkamp SE, Howard HJ, Roberts AJ, Grizzle J, Wise T. Relationships among concentrations of steroids, insulin-like growth factor-I, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. Biol Reprod 1994; 51: 971-981

Eckery DL, Moeller CL, Nett TM, Sawyer HR. Localisation and quantification of binding sites for FSH, LH, GH and IGF-1 in sheep ovarian follicles. Biol Reprod 1997; 57: 507-513

Edwards DR, Beaudry PP, Laing TD, Kowal V, Leco KJ, Lec PA, Lim SA. The roles of tissue inhibitors of metalloproteinases in tissue remodelling and cell growth. Internat J Obesity 1996; 20: 9-15

Elvin JA, Yan CN, Wang P, Nishimori K, Matzuk MM. Molecular characterisation of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 1999; 13: 1018-1034

Endo T, Aten RF, Wang F, Behrman HR. Coordinate induction and activation of metalloproteinase and ascorbate depletion in structural luteolysis. Endocrinol 1993; 133: 690-698

Engvall E. Laminin variants: why, where and when? Kidney International 1993; 43: 2-6

Eppig JJ, O'Brien MJ. Development *in vitro* of mouse oocytes from primordial follicles. Biol Reprod 1996; 54: 197-207

Eppig JJ, Peters A, Telfer EE, Wigglesworth K. Production of cumulus expansion enabling factor by mouse oocytes grown *in vitro*: preliminary characterisation of the factor. Mol Reprod Dev 1993; 34: 450-456

Eppig JJ, Schroeder AC. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation and fertilisation *in vitro*. Biol Reprod 1989; 41: 268-276

Eppig JJ. Intercommunication between mammalian oocytes and companion somatic cells. Bioessays 1991; 13: 569-574

Erickson BH. Development and senescence of the postnatal bovine ovary. J Anim Sci 1966; 25: 800-805

Erickson GF, Magoffin DA, Dyer CA, Hofeditz C. The ovarian androgen producing cells: a review of structure/function relationships. Endocr Rev 1985; 6: 371-399

Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Mol Reprod Dev 1995; 42: 437-442

Figueiredo JR, Hulshof SCJ, Thiry M, Van den Hurk R, Bevers MM, Nusgens B, Beckers JF. Extracellular matrix proteins and basement membrane: identification in bovine ovaries and significance for the attachment of cultured preantral follicles. Theriogenology 1995; 43: 845-858

Figueiredo JR, Hushof SCJ, Van den Hurk R, Nusgens B, Bevers MM, Ectors FJ, Beckers JF. Preservation of oocyte and granulosa cell morphology in bovine preantral follicles cultured *in vitro*. Theriogenology 1994; 41: 1333-1346

Findlay JK. An update on the roles of inhibin, activin and follistatin as local regulators of folliculogenesis. Biol Reprod 1993; 48: 15-23

Fowlkes JL, Enghild JJ, Suzuki K, Nagase H. Matrix metalloproteinases degrade insulin-like growth factor binding protein-3 in dermal fibroblast cultures. J Biol Chem 1994a; 269: 25,742-25,476

Fowlkes JL, Suzuki K, Nagase H, Thrailkill KM. Proteolysis of insulin-like growth factor binding protein-3 during rat pregnancy: a role for matrix metalloproteinases. Endocrinol 1994b; 135: 2810-2813

Fowlkes JL, Thrailkill KM, Serra DM, Suzuki K, Nagase H. Matrix metalloproteinases as insulin-like growth factor binding protein-degrading proteinases. Prog Growth Factor Res 1995; 6: 255-263

Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW. Beattie AE, Davis GH, Ritvos O. Mutations in an oocyte-derived growth differentiation factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nature Genetics 2000; 25: 279-283

Garcia R, Ballesteros LM, Hernandez-Perez O, Rosales AM, Espinosa R, Soto H, Diaz de Leon L, Rosado A. Metalloproteinase activity during growth, maturation and atresia in the ovarian follicles of the goat. Anim Reprod Sci 1997; 47: 211-218

Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol 1992; 119: 493-501

Ginther OJ, Knopf L, Kaestilic JP. Temporal associations among ovarian events in cattle during the oestrous cycles with two and three follicular waves. J Reprod Fertil 1989; 87: 223-230

Giudice, LC. Insulin-like growth factors and ovarian development. Endocr Rev 1992; 13: 641-669

Gomez E, Tarin JJ, Pellicer A. Oocyte maturation in humans: the role of gonadotropins and growth factors. Fertil Steril 1993; 60: 40-46

Gore-Langton RE, Daniel SA. Follicle-stimulating hormone and estradiol regulate antrum-like reorganisation of granulosa cells in rat preantral follicle cultures. Biol Reprod 1990; 43: 65-72

Gosden RG, Baird DT, Wadw JC, Webb R. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at –196 °C. Hum Reprod 1994; 9: 597-603

Gosden RG, Hunter RHF, Telfer E, Torrance C, Brown N. Physiological factors underlying the formation of ovarian follicular fluid. J Reprod Fertil 1988; 82: 813-825

Grimes RW, Guthrie HD, Hammond JM. Insulin-like growth factor binding protein-2 and –3 are correlated with atresia and preovulatory maturation in the porcine ovary. Endocrinol 1994; 135: 1966-2000

Grollman AP, Lehninger AL. Enzymic synthesis of L-ascorbic acid in different animal species. Arch Biochem Biophys 1957; 69: 458-467

Gutierrez CG, Armstrong DG, Campbell BK, Hogg CO, Webb R. Insulin-like growth factor (IGF) I production and expression of IGF -I and II by bovine granulosa and theca cells *in vivo* and *in vitro*. J Reprod Fertil Abstr Series 1996; 17: abstract 66

Gutierrez CG, Campbell BK, Armstrong DG, Webb R. Insulin-like growth factor-1 (IGF-1) production by bovine granulosa cells *in vitro* and peripheral IGF-1 measurement in cattle serum: an evaluation of IGF-binding protein extraction protocols. J Endocrinol 1997a; 153: 231-240

Gutierrez CG, Campbell BK, Webb R. Development of a long term bovine granulosa cell culture system; induction and maintenance of estradiol production, response to FSH and morphological characteristics. Biol Reprod 1997b; 56: 608-616

Gutierrez CG, Ralph JH, Telfer EE, Wilmut I, Webb R. Growth and antrum formation of bovine preantral follicles in long-term culture *in vitro*. Biol Reprod 2000; 62: 1322-1328

Hagglund AC, Ny A, Leonardsson G, Ny T. Regulation and localisation of matrix metalloproteinases in the mouse ovary during gonadotropin-induced ovulation. Endocrinol 1999; 140: 4351-4358

Harada M, Miyano T, Matsumara K, Osuki S, Miyake M, Kato S. Bovine oocytes from early antral follicles grow to meiotic competence *in vitro*; effect of FSH and hypoxanthine. Theriogenology 1997; 48: 743-755

Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A. Cell growth promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). J Cell Sci 1994; 107: 2373-2379

Henderson KM, Kiebom LE, McNatty KP, Lun S, Heath D. Gonadotrophin-stimulated cyclic AMP production by granulosa cells from Booroola x Romney ewes with and without a fecundity gene. J Reprod Fertil 1985; 75: 111-120

Hillier SG, Whitelaw PF, Smyth CD. Follicular oestrogen synthesis: the 'two cell, two-gonadotrophin' model revisited. Mol Cell Endocrinol 1994; 100: 51-54

Hillier SG, Yong EL, Illingworth PI, Baird DT, Schwall RH, Mason AJ. Effect of recombinant inhibin on androgen synthesis in cultured human thecal cells. Mol Cell Endocrinol 1991; 75: 79, 177

Himeno N, Kawamura N, Okamura H, Mori T, Fukomoto M, Midorikawa O. Collagen synthetic activity in rabbit ovary during ovulation and its blockage by indomethacin. Acta Obstet Gynaecol Jpn 1984; 36: 1930-1934

Hirao Y, Nagai T, Kubo M, Miyano T, Miyake M, Kato S. *In vitro* growth and maturation in pig oocytes. J Reprod Fertil 1994; 100: 333-339

Hirshfield AN. Development of follicles in the mammalian ovary. Internat Rev Cytol 1991; 124: 43-101

Hoch-Ligeti C, Bourne GH. Changes in the concentration and histological distribution of ascorbic acid in ovaries, adrenals and livers of rats during oestrous cycles. Br J Pathol 1948; 29: 400-407

Huang EJ, Manova K, Packer AI, Sanchez S, Bachvarova RF, Besmer P. The murine Steel Panda mutation affects kit ligand expression and growth of early ovarian follicles. Dev Biol 1993; 157: 100-109

Huet C, Monget P, Pisselet C, Monniaux D. Changes in ECM components and steroidogenic enzymes during growth and atresia of antral ovarian follicles in the sheep. Biol Reprod 1997; 56: 1025-1034

Hughes FM, Gorospe WC. Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. Endocrinol 1991; 129: 2415-2422

Hulmes DJS. The collagen superfamily- diverse structures and assemblies. Essays Biochem 1992; 27:49-67

Hulshof SCJ, Figueiredo JR, Beckers JF, Bevers MM, Van der Donk JA, Van den Hurk R. Effects of recombinant FSH, 17 β-oestradiol and their combination on bovine preantral follicles *in vitro*. Theriogenology 1995; 44: 217-226

Hyttel P, Fair T, Callesen H, Greve T. Oocyte growth, capacitation and final maturation in cattle. Theriogenology 1997; 47: 23-32

Iwamatsu T, Yanagamachi R. Maturation *in vitro* of ovarian oocytes of prepubertal and adult hamsters. J Reprod Fertil 1975; 45: 83-90

Jeffrey JJ, Martin GR. The role of ascorbic acid in the biosynthesis of collagen. I. Ascorbic acid requirement by embryonic chick tibia in tissue culture. Biochem Biophys Acta 1966; 121: 269-280

Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: Biological actions. Endocr Rev 1995; 16: 3-34

Kerr JFR, Winterford CM, Harmon BV. Morphological criteria for identifying apoptosis. In Celis JE (ed) Cell Biology: A Laboratory Handbook Academic Press, San Diego, CA, USA 1994; 319-329

Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Brit J Cancer 1972; 26: 239-257

Kitabchi AE. Ascorbic acid in steroidogenesis. Nature 1967; 215: 1385-1386

Kleiner DE, Stetler-Stevenson WG. Structural biochemistry and activation of the matrix metalloproteinases. Current Opinion in Cell Biol 1993; 5: 891-897

Knight PG, Glister C. Potential local regulatory functions of inhibins, activins and follistatin in the ovary. Reproduction 2001; 121: 503-512

Kramer MM, Harman MT, Brill AK. Disturbances of reproduction and ovarian changes in the guinea-pig in relation to Vitamin C deficiency. Am J Physiol 1933; 106: 611-622

Langhout DJ, Spicer LJ, Geisert RD. Development of a culture system for bovine granulosa cells: effects of growth hormone, estradiol and gonadotrophins on cell proliferation, steroidogenesis and protein synthesis. J Anim Sci 1991; 69: 3321-3334

Leeuwenberg BR, Hurst PR, McNatty KP. Expression of IGF-1 messenger-RNA in the ovine ovary. J Mol Endocrinol 1995; 15: 251-258

Liu XJ, Malkowski M, Guo Y, Erickson G, Shimasaki S, Ling NC. Development of specific antibodies to rat insulin-like growth factors-binding proteins (IGFBP-2 to 6): analysis of IGFBP production by granulosa cells. Endocrinol 1993; 132: 1176-1183

Lobb DK, Dorrington J. Intraovarian regulation of follicular development. Anim Reprod Sci 1992; 28: 343-354

Logan A, Hill DJ. Bioavailability: Is this a key event in regulating the actions of peptide growth factors? J Endocrinol 1992; 134: 157-161

Luciano AM, Pappalardo A, Ray C, Peluso JJ. Epidermal growth factor inhibits large granulosa cell apoptosis by stimulating progesterone synthesis and regulating the distribution of intracellular free calcium. Biol Reprod 1994; 51: 646-654

Luck MR, Jeyaseelan I, Scholes RA. Ascorbic acid and fertility. Biol Reprod 1995; 52: 262-266

Luck MR, Zhao Y. Identification and measurement of collagen in the bovine corpus luteum and its relationship with ascorbic acid and tissue development. J Reprod Fertil 1993; 99: 647-652

Luck MR. The gonadal extracellular matrix. Oxford Rev Reprod Biol 1994; 16: 33-85

Lussier JG, Matton P, Dufour JJ. Growth rates of follicles in the ovary of the cow. J Reprod Fertil 1994; 81: 301-307

Majno G, Joris I. Apoptosis, oncosis and necrosis. An overview of cell death. Am J Pathol 1995; 146: 3-15

Manuel Silva J, Price CA. Effect of follicle-stimulating hormone on steroid secretion and messenger ribonucleic acids encoding cytochromes P450 aromatase and cholesterol side-chain cleavage in bovine granulosa cells *in vitro*. Biol Reprod 2000; 62: 186-191

Martimbeau S, Tilly JL. Physiological cell death in endocrine-dependent tissues: an ovarian perspective. Clin Endocrinol 1997; 46: 241-254

Mason HD, Cwyfan-Hughes SC, Heinrich G, Franks S, Holly JM. Insulin-like growth factor (IGF) I and II, IGF-binding proteins, and IGF-binding protein proteases are produced by theca and stroma of normal and polycystic human ovaries. J Clin Endocrinol Metab 1996; 81: 276-284

Mather JP, Moore A, Li RH. Activins, inhibins and follistatins: further thoughts on a growing family of regulators. Proc Soc Exp Biol Med 1997; 215: 209-222

Matzuk MM. Revelations of ovarian follicle biology from gene knockout mice. Mol Cell Endocrinol 2000; 163: 61-66

McGee E, Spears N, Minami S, Hsu SY, Chun SY, Billig H, Hsueh AJ. Preantral ovarian follicles in serum-free culture: suppression of apoptosis after activation of the cyclic guanosine 3', 5'-monophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. Endocrinol 1997; 138: 2417-2424

McGrath SA, Equela AF, Lee SJ. Oocyte specific expression of growth/ differentiation factor-9. Mol Endocrinol 1995; 9: 131-136

McIntush EW, Smith MF. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in ovarian function. Rev Reprod 1998; 3: 23-30

McNatty KP, Heath DA, Lundy T, Fidler AE, Quirke L, O'Connell A, Smith P, Groome N, Tisdall DJ. Control of early follicular development. J Reprod Fertil Suppl 1999; 54: 3-16

McNatty KP, Lun S, Heath DA, Ball K, Smith P, Hudson NL, McDiarmid J, Gibb M, Henderson KM. Differences in ovarian activity between Booroola x Merino ewes which were homozygous, heterozygous and non-carriers of a major gene influencing their ovulation rate. J Reprod Fertil 1986; 77: 193-205

McNatty KP, Makris A, de Grazia C, Osathanondh R, Ryan KJ. The production of progesterone, androgens, and oestrogens by human granulosa cells, thecal tissue and stromal tissue *in vitro*. J Clin Endocrinol Metab 1979; 49: 687-699

Mizunuma H, Liu X, Andoh K, Abe Y, Kobayashi J, Yamada K, Yokota H, Ibuki Y, Hasegawa Y. Activin from secondary follicles causes small preantral follicles to remain dormant at the resting stage. Endocrinol 1999; 140: 37-42

Monget P, Monniaux D, Pisselet C, Durand P. Changes in insulin-like growth factor-I (IGF-1), IGF-II, and their binding proteins during growth and atresia of ovine ovarian follicles. Endocrinol 1993; 132: 1438-1446

Monniaux D, de Reviers MM. Quantitative autoradiographic study of FSH binding sites in prepubertal ova of three strains of rats. J Reprod Fertil 1989; 85: 151-162

Monniaux D, Monget P, Pisselet C, Fontaine J, Elsen JM. Consequences of the presence of the Booroola F gene on the intraovarian insulin-like growth factor system and terminal follicular maturation in Mérinos d'Arles ewes. Biol Reprod 2000; 63: 1205-1213

Motlik J, Fulka J. Factors affecting meiotic competence in pig oocytes. Theriogenology 1986; 25: 87-96

Motro B, Bernstein A. Dynamic changes in ovarian c-kit and Steel expression during the estrous reproductive cycle. Dev Biol 1993; 197: 69-79

Murray AA, Molinek MD, Baker SJ, Kojima FN, Smith MF, Hillier SG, Spears N. Ascorbic acid promotes follicle integrity and survival in intact murine ovarian follicles *in vitro*. Reproduction 2001; 121: 89-96

Nagase H. Activation mechanisms of matrix metalloproteinases. J Biol Chem 1997; 378: 151-160

Nakano R, Mizuno T, Katayama K, Tojo S. Growth of ovarian follicles in rats in the absence of gonadotrophins. J Reprod Fertil 1975; 45: 545-546

Nayudu PL, Osborn SM. Factors influencing the rate of preantral and antral growth of mouse ovarian follicles *in vitro*. J Reprod Fertil 1992; 95: 349-362

Newton H, Picton H, Gosden RG. *In vitro* growth of oocyte-granulosa cell complexes isolated from cryopreserved ovine tissue. J Reprod Fertil 1999; 115: 141-150

Nothnick WB, Curry TE. Divergent effects of interleukin-1 beta on steroidogenesis and matrix metalloproteinase inhibitor expression and activity in cultured rat granulosa cells. Endocrinol 1996; 137: 3784-3790

Oliver JE, Aitman TJ, Powell JF, Wilson CA, Clayton RN. Insulin-like growth factor I gene expression in the rat ovary is confined to the granulosa cells of developing follicles. Endocrinol 1989; 124: 2671-2679

Padh H. Vitamin C: newer insights into its biochemical functions. Nutr Rev 1991; 49: 65-70

Pawshe CH, Apparao KBC, Totey SM. Effect of insulin-like growth factor I and its interaction with gonadotropins on *in vitro* maturation and embryonic development, cell proliferation and biosynthesis activity of cumulus-oocyte complexes and granulosa cells in buffalo. Mol Reprod Dev 1998; 49: 277-285

Pederson T. Follicle kinetics in the ovary of the cyclic mouse. Acta Endocrinologica 1970; 64: 304-323

Peluso J, Luciano AM, Pappalardo A, White BA. Cellular and molecular mechanisms that mediate insulin-dependent rat granulosa cell mitosis. Biol Reprod 1995; 52: 124-130

Perks CM, Dennining-Kendall PA, Gilmour RS, Wathes DC. Localisation of messenger ribonucleic acids for insulin-like growth factor I (IGF-I), IGF-II and the

type I IGF receptor in the ovine ovary throughout the estrous cycle. Endocrinol 1995; 136: 5266-5273

Perks CM, Peters AR, Wathes DC. Follicular and luteal expression of insulin-like growth factors I and II and the type I IGF receptor in the bovine ovary. J Reprod Fertil 1999; 116: 157-165

Peters H, Byskov AG, Lintern-Moore S, Faber M. Proceedings: Follicle growth initiation in the immature mouse ovary: extraovarian or intraovarian control? J Reprod Fertil 1973; 35: 619-620

Pfeffer F, Casenueva E, Kamar J, Guerra A, Perichart O, Vadillo-Ortega F. Modulation of 72-kilodalton type IV collagenase (matrix metalloproteinase-2) by ascorbic acid in cultured human amnion-derived cells. Biol Reprod 1998; 59: 326-329

Pinnell SR. Regulation of collagen synthesis by ascorbic acid: a review. Yale J Biol Med 1985; 58: 553-559

Piper LR, Bindon BM. The Booroola Merino and the performance of medium non-Peppin crosses at Armidale. In Piper LR, Bindon BM, Nethery RD (eds), The Booroola Merino; Melbourne, Australia: CSI-RO; 1982: 9-20

Qu J, Godin PA, Nisolle M, Donnez J. Expression of receptors for insulin-like growth factor-1 and transforming growth factor-β in human follicles. Mol Hum Reprod 2000; 6: 137-145

Qvist R, Blackwell LF, Bourne H, Brown JB. Development of mouse ovarian follicles from primary to preovulatory stages *in vitro*. J Reprod Fertil 1990; 89: 169-180

Rajakoski E. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical and left-right variation. Acta Endocrinologica Suppl 1960; 52: 1-68

Ralph JH, Telfer EE, Wilmut I. Bovine cumulus cell expansion does not depend on an oocyte secreted factor. Mol Reprod Dev 1995a; 42: 248-253

Ralph JH, Telfer EE, Wilmut I. *In vitro* growth of bovine preantral follicles and the influence of FSH on follicular and oocyte diameters. J Reprod Fertil Abstr Series 1995b; 15: abstract 6

Ralph JH. Factors affecting follicle and oocyte development in cattle. Thesis 1996, University of Edinburgh.

Reich R, Daphna-Iken D, Chun SY, Popliker M, Slager R, Adelmann-Grill BC, Tsafriri A. Preovulatory changes in ovarian expression of collagenases and tissue metalloproteinase inhibitor mRNA: role of eicosanoids. Endocrinol 1991; 129: 1869-1891

Revel F, Mermillod P, Peynot N, Renard JP, Heyman Y. Low developmental capacity of *in vitro* matured and fertilized oocytes from calves compared with that of cows. J Reprod Fertil 1995; 103: 115-120

Richards JS, Fitzpatrick SL, Clemens JW, Morris JK, Allsiton T, Sirois J. Ovarian cell differentiation: a cascade of multiple hormones, cellular signals and regulated genes. Rec Prog Horm Res 1995; 50: 223-254

Richardson MC, Davies DW, Watson RH, Dunsford ML, Inman CB, Masson GM. Cultured human granulosa cells as a model for corpus luteum function: relative roles of gonadotrophin and low density lipoprotein studied under defined culture conditions. Hum Reprod 1992; 7: 12-18

Riley SC, Leask R, Chard T, Wathen NC, Calder A, Howe DC. Secretion of matrix metalloproteinase-2, matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases into the intrauterine compartments during early pregnancy. Mol Hum Reprod 1999; 5: 376-381

Roberts AJ, Skinner MK. Transforming growth factor-alpha and –beta differentially regulate growth and steroidogénesis of bovine thecal cells during antral follicle development. Endocrinol 1991; 129: 2041-2048

Rodgers RJ, Irving-Rodgers HF, van Wezel IL. Extracellular matrix in ovarian follicles. Mol Cell Endocrinol 2000; 163: 73-79

Rodgers HF, Irvine CM, van Wezel IL, Lavranos TC, Luck MR, Sao Y, Ninomiya Y, Rodgers RJ. Distribution of the α1 to α6 chains of Type IV collagen in bovine follicles. Biol Reprod 1998; 59: 1334-1341

Rodgers HF, Lavranos TC, Vella CA, Rodgers RJ. Basal lamina and other extracellular matrix produced by bovine granulosa cells in anchorage-independent culture. Cell Tissue Res 1995; 282: 463-471

Rose RC, Bode AM. Biology of free radical scavengers: an evaluation of ascorbate. FASEB J 1993; 7: 1135-1142

Rose UM, Hanssen RG, Kloosterboer HJ. Development and characterization of an *in vitro* ovulation model using mouse ovarian follicles. Biol Reprod 1999; 61: 503-11

Roy SK, Greenwald GS. Hormonal requirements for the growth and differentiation of hamster preantral follicles in long term culture. J Reprod Fertil 1989; 87: 103-114

Roy SK, Treacy BJ. Isolation and long term culture of human preantral follicles. Fertil Steril 1993; 59 (suppl 4): 783-790

Saha S, Shimizu M, Geshi M, Izaike Y. *In vitro* culture of bovine preantral follicles. Anim Reprod Sci 2000; 63: 27-39

Sanyal S and Datta S. Effect of ascorbic acid in *in vitro* rat adrenal and ovarian steroidogenesis. Indian J Exp Biol 1979; 17: 86-88

Sasano H, Okamoto M, Mason JI, Simpson ER, Mandelson CR, Sasano N, Silverberg SG. Immunolocalisation of aromatase, 17α-hydroxylase and side-chain cleavage cytochromes P-450 in the human ovary. J Reprod Fertil 1989; 85: 163-169

Saumande J. Culture of bovine granulosa cells in a chemically defined serum-free medium: the effect of insulin and fibronectin on the response to FSH. J Steroid Biochem and Mol Biol 1991; 38: 189-196

Savio JD, Keenan L, Boland MP, Roche JF. Pattern of growth of dominant follicles during the oestrous cycle of heifers. J Reprod Fertil 1988; 83: 663-671

Schams D, Berisha B, Kosmann M, Einspanier R, Amselgruber WM. Possible role of growth hormone, IGFs, and IGF-binding proteins in the regulation of ovarian function in large farm animals. Domestic Anim Endocrinol 1999; 17: 279-285

Schoot DC, Coellingh Bennink HJT, Mannaert BMJL, Lamberts SWJ, Bouchard P, Fauser BCJM. Human recombinant follicle-stimulating hormone induces growth of preovulatory oocytes without concomitant increase in androgen and oestrogen biosynthesis in a woman with isolated gonadotrophin deficiency. J Clin Endocrinol Metab 1992; 74: 1471-1473

Sekar N, Lavoie HA, Veldhuis JD. Concerted regulation of steroidogenic acute regulatory gene expression by luteinizing hormone and insulin (or insulin-like growth factor I) in primary cultures of porcine granulosa-luteal cells. Endocrinol 2000; 141: 3983-3992

Shimasaki S, Gao L, Shimonaka M, Ling N. Isolation and molecular cloning of insulin-like growth factor binding protein-6. Mol Endocrinol 1991; 5: 938-948

Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S, Ueno N, Sampath K, Chang RJ, Erickson GF. A functional bone morphogenetic protein system in the ovary. Proc Nat Acad Sci USA 1999; 96: 7282-7287

Sirois J, Fortune JE. Ovarian follicular dynamics during the oestrous cycle in heifers monitored by real-time ultrasonography. Biol Reprod 1988; 39: 308-317

Smith GW, Goetz TL, Anthony RV, Smith MF. Molecular cloning of an ovine ovarian tissue inhibitor of metalloproteinases: ontogeny of mRNA expression and in situ localisation within preovulatory follicles and luteal tissue. Endocrinol 1994; 134: 344-352

Smith MF, Gutierrez CG, Armstrong DG, Webb R. Effect of LH on secretion of MMP by bovine thecal cells. J Reprod Fertil Abstr Series 1997; 19: abstract 169

Smith MF, McIntush EW, Ricke WA, Kojima FN, Smith G. Regulation of ovarian extracellular matrix remodelling by metalloproteinases and their tissue inhibitors: effects on follicular development, ovulation and luteal function. J Reprod Fertil Suppl 1999; 54: 367-381

Souza CJH, MacDougall C, Campbell BK, McNeilly AS, Baird DT. The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. J Endocrinol 2001; 169: R1-R6

Spears N, Boland NI, Murray AA, Gosden RG. Mouse oocytes derived from *in vitro* grown primary ovarian follicles are fertile. Biol Reprod 1994; 48: 798-806

Spears N, Murray AA, Allison V, Boland NI, Gosden RG. Role of gonadotrophins and ovarian steroids in the development of mouse follicles *in vitro*. J Reprod Fertil 1998; 113: 19-26

Spicer LJ, Alpizar E, Echternkamp SE. Effects of insulin, insulin-like growth factor-I and gonadotrophins on bovine granulosa cell proliferation, progesterone production, estradiol production and (or) insulin-like growth factor production *in vitro*. J Anim Sci 1993; 71: 1232-1241

Spicer LJ, Stewart RE, Alvarez P, Francisco CC, Keefer BE. Insulin-like growth factor binding protein-2 and –3: their biological effects in bovine thecal cells. Biol Reprod 1997; 56: 1458-1465

Stewart RE, Spicer LJ, Hamilton TD, Keefer BE. Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis of bovine thecal cells: involvement of glucose and receptors for insulin-like growth factor I and luteinizing hormone. J Anim Sci 1995; 73: 3719-3731

Spicer LJ, Echternkamp SE. The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. Dom Anim Endocrinol 1995; 12: 223-245

Telfer EE, Binnie JP, Jordan LB. Effect of follicle size on the onset of apoptotic cell death in cultured bovine ovarian follicles. Theriogenology 1998a; 49: 357

Telfer EE, Binnie JP, McCaffery F, Campbell BK. Development of immature follicles from porcine and bovine ovaries. Mol Cell Endocrinol 2000; 163: 117-123

Telfer EE. *In vitro* models for oocyte development. Theriogenology 1998b; 49: 451-460

Thrailkill KM, Quarles LD, Nagase H, Suzuki K, Serra DM, Fowlkes JL. Characterisation of insulin-like growth factor binding protein 5-degrading proteinases produced throughout murine osteoblast differentiation. Endocrinol 1995; 136: 3527-3533

Tilly JL and Tilly KI. Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. Endocrinol 1995; 136: 242-252

Tilly JL, Billig H, Kowalski KI, Hsueh AJW. Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles by a tyrosine kinase-dependent mechanism. Mol Endocrinol 1992; 6: 1942-1950

Tilly JL, Kowalski KI, Johnson AL, Hsueh AJ. Involvement of apoptosis in ovarian follicular atresia and post ovulatory regression. Endocrinol 1991; 129: 2799-2801

Tisdall DJ, Quirke LD, Smith P, McNatty KP. Expression of the ovine stem cell factor gene during folliculogenesis in late fetal and adult ovaries. J Mol Endocrinol 1997; 18: 127-135

Tonetta SA, diZerega GS. Intragonadal regulation of follicular maturation. Endocrine Rev 1989; 10: 205-229

Torrance C, Telfer E, Gosden RG. Quantitative study of the development of isolated mouse preantral follicles in collagen gel culture. J Reprod Fertil 1989; 87: 367-374

Tsuji M , Ito Y, Terada N, Mori H. Ovarian aromatase action in scorbutic mutant rats unable to synthesise ascorbic acid. Acta Endocrinol (Copenh) 1989; 121: 595-602

Van den Hurk R, Abir R, Telfer EE, Bevers MM. Primate and bovine immature oocytes and follicles as sources of fertilizable oocytes. Hum Reprod Update 2000; 6: 457-474

Van den Hurk R, Bevers MM, Beckers JF. *In vivo* and *in vitro* development of preantral follicles. Theriogenology 1997; 47: 73-82

van Wezel IL, Rodgers RJ. Morphological characterisation of bovine primordial follicles and their environment *in vivo*. Biol Reprod 1996; 55: 1003-1011

Vanderhyden BC, Caron PJ, Buccione R, Eppig JJ. Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. Developmental Biol 1990; 140: 307-317

Vanderhyden BC, Telfer EE, Eppig JJ. Mouse oocytes promote proliferation of granulosa cells from preantral and antral follicles *in vitro*. Biol Reprod 1992; 46: 1196-1204

Wandji SA, Eppig JJ, Fortune JE. FSH and growth factors affect the growth and endocrine function *in vitro* of granulosa cells of bovine preantral follicles. Theriogenology 1996; 45: 817-832

Wandji SA, Pelletier G, Sirard MA. Ontogeny and cellular localisation of ^{125}I -labelled insulin-like growth factor-I, ^{125}I -labelled follicle-stimulating hormone, and ^{125}I -labelled human chorionic gonadotropin binding sites in ovaries from bovine fetuses and neonatal calves. Biol Reprod 1992; 47: 814-822

Wandji SA, Wood TL, Crawford J, Levison SW, Hammond JM. Expression of mouse ovarian insulin-like growth factor system components during follicular development and atresia. Endocrinol 1998; 139: 5205-5214

Wathes DC, Perks CM, Davis AJ, Denning-Kendall PA. Regulation of insulin-like growth factor-1 and progesterone synthesis by insulin and growth hormone in the ovine ovary. Biol Reprod 1995; 53: 882-889

Watt FM. The extracellular matrix and cell shape. Trends Biochem Sci 1986; 11: 482-585

Webb R, Armstrong DG. Control of ovarian function; effect of local interactions and environmental influences on follicular turnover in cattle: a review. Livestock Prod Sci 1998; 53: 95-112

Webb R, Baxter G, McBride D, Nordblom GD, Shaw MPK. The measurement of testosterone and oestradiol-17β using iodinated tracers and incorporating an affinity chromatography extraction procedure. J Steroid Biochem 1985; 23: 1043-1051

Webb R, Gosden RG, Telfer EE, Moor RM. Factors affecting folliculogenesis in ruminants. Anim Sci 1999; 68: 257-284

Wilson T, Wu XY, Jeungel JL, Ross IK, Lumsden JM, Lord EA, Dodds KG, Walling GA, McEwan JC, O'Connell AR, McNatty KP, Montgomery GW. Hightly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein 1B receptor (ALK-6) that is expressed in both oocytes and granulosa cells. Biol Reprod 2001; 64: 1225-1235

Wise T. Biochemical analysis of bovine follicular fluid: albumin, total protein, lysosomal enzymes, ions, steroids and ascorbic acid content in relation to follicle size, rank, atresia classification and day of estrus cycle. J Anim Sci 1987; 64: 1153-1169

Wright CS, Hovatta O, Margara R, Trew G, Winston RM, Franks S, Hardy K. Effects of follicle stimulating hormone and serum substitution on the *in vitro* growth of human ovarian follicles. Hum Reprod 1999; 14: 1555-1562

Wu J, Emery BR, Carrell DT. *In vitro* growth, maturation, fertilisation and embryonic development of oocytes from porcine preantral follicles. Biol Reprod 2001; 64: 375-381

Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980; 284: 555-556

Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H, Nishikawa SI. Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development. Dev Biol 1997; 184: 122-137

Yoshimura Y, Ando M, Nagamatsu S, Iwashita M, Adachi T, Sueoka K, Miyazaki T, Kuji N, Tanaka M. Effects of insulin-like growth factor-I on follicle growth, oocyte maturation, and ovarian steroidogenesis and plasminogen activator activity in the rabbit. Biol Reprod 1996; 55: 152-160

Yuan W, Lucy MC, Smith MF. Messenger ribonucleic acid for insulin-like growth factors -I and -II, insulin-like growth factor-binding protein -2, gonadotropin receptors, and steroidogenic enzymes in porcine follicles. Biol Reprod 1996; 55: 1045-1054

Zakeri Z, Bursch W, Tenniswood M, Lockshin RA. Cell death: programmed, apoptosis, necrosis, or other? Cell Death and Differentiation 1995; 2: 87-96

Zeleznik AJ, Hillier SG. The ovary: endocrine function. In Hillier SG, Kitchener HC, Neilson JP (eds) Scientific essentials of reproductive medicine. WB Saunders company Ltd; 1996: 133-147

Zhao H, Luo L, Liu Y. The effect of insulin-like growth factor-I on steroidogenesis and ultrastructures of cultured human granulosa cells *in vitro*. Zhonghua Fu Chan Ke Za Zhi 1998; 33: 546-548

Zhao Y, Luck MR. Bovine granulosa cells express ECM proteins and their regulators during luteinization in culture. Reprod Fertil Dev 1996; 8: 259-226

Zhou J, Adesanya OO, Vatzias G, Hammond JM, Bondy CA. Selective expression of insulin-like growth factor system components during porcine ovary follicular selection. Endocrinol 1996; 137: 4893-4901

Zhou J, Bievre M, Bondy CA. Reduced GLUT1 expression in IGF1 -/- null oocytes and follicles. Growth Horm Res 2000; 10: 111-117

Zhou J, Kumar TR, Matzuk MM, Bondy C. Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. Mol Endocrinol 1997; 11: 1924-1933

Culture of Bovine Preantral Follicles in a Serum-Free System: Markers for Assessment of Growth and Development[1]

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ABSTRACT

Satisfactory development of bovine follicles in vitro remains elusive. This study used a serum-free system to evaluate the effects of insulin-like growth factor-1 (IGF-1) on bovine preantral follicles in culture and to identify the activity of gelatinase matrix metalloproteinases (MMPs) and their endogenous inhibitors (TIMPs) in vitro to assess their potential as markers of development. Preantral follicles were cultured for 6 days in serum-free medium containing insulin and IGF-1 (10 ng/ml). No difference was observed in follicular growth, health, or antrum formation between IGF-1-treated follicles and controls. However, IGF-1 had a negative effect ($P < 0.01$) on oocyte size and granulosa cell proliferation. When MMP-9 was secreted, the probability of follicles having healthy granulosa or theca cells at the end of the culture period was 0.85 and 0.60, respectively. If TIMP-1 was released, the probability of follicles having healthy somatic cells was 0.79. When TIMP-2 was detected, the probability of granulosa and theca cell health was 0.78 and 0.67, respectively. These results demonstrate no positive effects of IGF-1 on bovine follicles in this system. Furthermore, MMP-9 and TIMPs are related to follicular health and, therefore, can be used as markers of follicular development.

follicle, granulosa cells, ovary, theca cells

INTRODUCTION

The number of oocytes in the ovary at birth varies enormously between species, ranging from tens of thousands in mice to millions in humans and domestic species [1]. Of these oocytes, less than 1% will eventually ovulate [2]. In cattle, procedures for in vitro maturation and fertilization have progressed substantially during the past decade, leading to increased availability of zygotes, embryos, and calves for breeding and research purposes [3]. However, this technology has limitations, because oocytes cannot be harvested from the large population of hormone-insensitive, preantral follicles. Development of a culture system that can support preantral and, eventually, primordial follicles to a stage at which the oocytes can be matured and fertilized in vitro would help to provide large amounts of homogeneous oocytes. Such a system would also allow the identification of factors necessary for normal follicular development and acquisition of oocyte developmental competence.

Culture systems developed for rodent follicles [4–8] have led to the production of developmentally competent oocytes after culture of whole preantral follicles or their granulosa-oocyte complexes. Advances have been made in transferring aspects from the rodent models to develop culture systems for porcine preantral follicles [9], but progress with other domestic species has been much slower. Recently, a culture system for sheep preantral follicles was described [10] that achieved antrum formation and estradiol production and maintained healthy oocytes and cumulus cells during a 6-day culture period. However, this system failed to produce germ cells with high developmental potential. Similarly, for bovine follicles, culture has been terminated long before the preovulatory stage was reached [11–14]. In cattle, large ovaries, less densely packed follicles, fibrous stromal tissue, large follicle size and slow follicular growth have all played a role in delaying the development of successful isolation and culture techniques. Recently, however, accelerated growth of bovine preantral follicles to the antral stage has been achieved over a period of as many as 28 days [11], with FSH, epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1) having a positive effect on follicular growth and development.

Insulin-like growth factors, and particularly IGF-1, have been identified as being important paracrine regulators of ovarian function. Previous studies have helped to determine the actions of IGFs in antral follicles, which are thought to vary according to species and the interaction of gonadotropins [15]. For example, granulosa cells from bovine antral follicles have increased proliferation and estradiol production in response to IGF-1 [16]. However, the precise role of the IGF system during the earlier stages of follicular development is poorly understood.

Previously, culture systems for cattle preantral follicles have resulted in loss of theca cells [11, 13] or rupture of the basement membrane [11] in some follicles. During follicular growth, turnover and reconstruction of the basement membrane is facilitated by matrix metalloproteinases (MMPs), which are zinc- and calcium-dependent enzymes that can degrade the protein components of the extracellular matrix [17]. The MMPs are regulated by tissue inhibitors of metalloproteinases (TIMPs) and are also responsible for reconstruction of the basement membrane at the time of ovulation and during corpus luteum formation [18]. The MMPs involved in the breakdown of collagen IV (i.e., a major constituent of the basement membrane) are the gelatinases MMP-2 and MMP-9. The main follicular sources of MMPs and TIMPs are thought to be the granulosa and theca cells [18, 19], but TIMP protein has also been found in oocytes [20].

The present study used a serum-free culture system [11] to investigate the effects of IGF-1 on several aspects of bovine preantral and early antral development in vitro. In

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Received: 1 November 1999.
First decision: 30 November 1999.
Accepted: 1 March 2000.

ISSN: 0006-3363. http://www.biolreprod.org

addition, secretion of MMPs and TIMPs by cultured follicles was identified, and using morphological comparisons, these factors were assessed for use as markers of follicular development.

MATERIALS AND METHODS

Isolation of Preantral Follicles

Bovine ovaries from random stages of the estrous cycle were obtained from an abattoir and transported at 25–30°C. Beneath a laminar flow hood, ovaries were rinsed with 70% alcohol, and fine slices of ovarian cortex were taken using a scalpel and placed in Liebovitz's medium (GIBCO BRL, Life Technologies Ltd., Paisley, Renfrewshire, UK) supplemented with sodium pyruvate (2 mM), glutamine (2 mM), BSA (3 mg/ml), penicillin G (75 μg/ml), and streptomycin (50 μg/ml). All chemicals were from Sigma Chemicals (Poole, Dorset, UK) unless otherwise stated. In a petri dish under the dissecting microscope, preantral follicles (100–200 μm) were isolated from the cortical slices using fine, 25-gauge needles attached to syringe barrels. Follicles with an intact basement membrane and an even distribution of granulosa and theca layers were selected for culture.

Culture of Preantral Follicles

For the control group, preantral follicles were cultured individually in 96-well plates (Bibby Sterilin Ltd., Stone, Staffs, UK) in 250 μl of culture medium (McCoy's 5a medium with bicarbonate [Sigma]) supplemented with Hepes (20 mM), BSA (0.1%), L-glutamine (3 mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), transferrin (2.5 μg/ml), selenium (4 ng/ml), androstenedione (10^{-7} M) and insulin (10 ng/ml). For the treatment group, 10 ng/ml of the analogue Long R3 IGF-1 (Gropep Pty Ltd., Adelaide, Australia), which does not bind to IGF-binding proteins, was added to the control medium. Plates were incubated for 6 days in a sterile, humidified air atmosphere with 5% CO_2 at 37°C. Follicular diameters were measured under the dissection microscope on Days 0, 2, 4, and 6. Half the medium was replaced every second day, and this conditioned medium was stored at −20°C for subsequent MMP/TIMP analysis.

Histological Assessment of Follicles

At the end of the culture period, follicles were fixed overnight in Bouin's solution and dehydrated in ethanol. Absolute ethanol was replaced with cedar wood oil for a minimum of 24 h, and then the oil was cleared from the follicles using toluene for 30 min. Follicles were embedded in paraffin wax (60°C), with changes every hour for 4 h to remove all traces of toluene. The samples were sectioned (6 μm), mounted on gelatin-coated slides, and then allowed to dry overnight at 37°C before staining with hematoxylin-and-eosin.

Histological measurements and observations were made under the light microscope with a crossed micrometer (Graticules Ltd., Tonbridge, Kent, UK). The section containing the oocyte nucleolus or, if this was absent, the largest cross-section of the oocyte was used for observations and measurements. Follicular and oocyte sizes were measured, and proliferation was assessed by counting the number of granulosa and theca cell layers. Granulosa cell death was measured by counting the number of pyknotic cells and then expressing them as a percentage of the total number of granulosa cells; atretic follicles were defined as being those with more than 5% pyknotic nuclei [21]. Theca cell health was assessed in two ways: by the level of pyknosis, as defined earlier; and by the number of layers present, with less than three layers indicating theca cell degeneration. Oocyte quality was assessed on a scale of 0–2, with 0 indicating an absent or severely misshapen oocyte with no germinal vesicle and obviously degenerate, 1 indicating a misshapen oocyte, and 2 indicating a morphologically normal oocyte with an intact germinal vesicle.

Detection of MMP-2 and MMP-9 Secretion by Gelatin Zymography

The MMP-2 and MMP-9 activities were detected by gelatinase zymography according to the method described by Riley et al. [22]. In brief, 100-μl samples of culture medium were dialyzed against distilled H_2O in small tube-O-dialyzers (Chemicon International, London, UK) before being lyophilized and reconstituted in 7.5 μl, 0.1% SDS in H_2O. Samples were separated by SDS-PAGE using 7.5% gels containing 1 mg/ml gelatin on a minigel apparatus (Bio-Rad, Hemel Hempstead, UK). Gels were washed (twice for 15 min in 2.5% (v/v) Triton X-100, and twice for 2 min in 10× Tris-buffered saline [TBS]), then incubated overnight at 37°C in digestion buffer (50 mM Tris; 0.2 M NaCl; 5 mM $CaCl_2$, 1 M $ZnCl_2$; 0.02% [v/v] Brij-35) Gels were stained for 3 h at 23°C with 0.5% Coomassie Blue R250 in 30% methanol/10% glacial acetic acid in H_2O and then destained (staining solution with Coomassie Blue omitted). Destaining revealed white bands where gelatin was degraded by gelatinase activity. The MMP-2 and MMP-9 were identified by comparison with molecular weight markers and control standards of human amniotic fluid collected during labor at term [22].

Detection of TIMP-1 and TIMP-2 Secretion by Reverse Zymography

The TIMP activity was detected using a commercial kit (University Technologies Inc., Calgary, Canada) according to the method described by Riley et al. [22]. Samples were lyophilized and reconstituted as before and separated according molecular weight by PAGE using 12% gels containing gelatin (1 mg/ml) and a preparation of MMP-2 (conditioned medium from BHK-21 cells that constitutively express MMP-2; University Technologies). The gels were washed (50 mM Tris, 5 mM $CaCl_2$, 2.5% [v/v] Triton X-100) for 2.5 h at 23°C, then incubated in digestion buffer (50 mM Tris, 5 mM $CaCl_2$) overnight at 37°C. After staining and destaining (as in zymography), TIMPs were represented by discrete, dark bands on the gel where inhibition of MMP-induced degradation of the gelatin substrate had occurred. The TIMPs were identified by comparison with molecular weight markers and control standards of conditioned medium containing mouse TIMP-1, -2, and -3 expressed by transfected BHK cells (University Technologies).

Statistical Analyses

Mean follicular diameters from the two experimental groups on Day 6 were compared using a 2-sample *t*-test. In addition, mean follicular growth rates from Days 0 to 6 were compared within groups using the same test. Oocyte diameters from freshly isolated and cultured preantral follicles were compared using a one-way ANOVA, with subsequent *t*-tests to allow for individual comparisons between

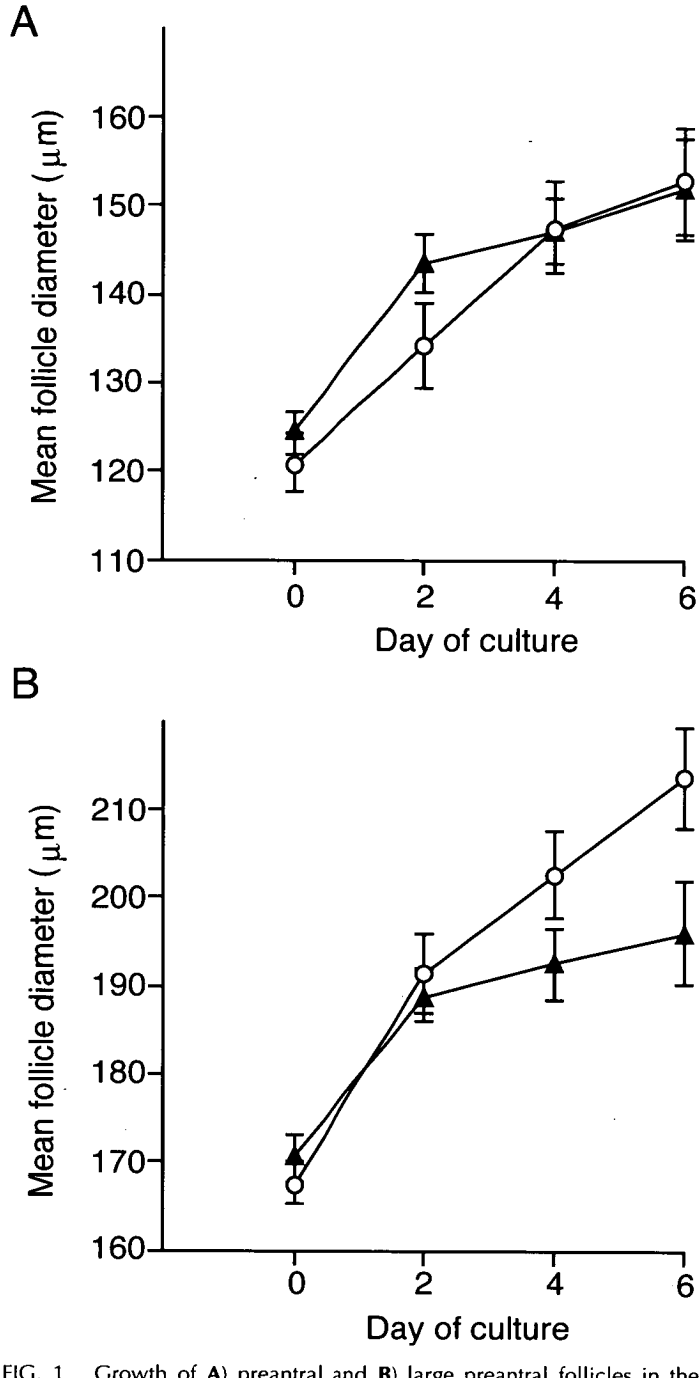

FIG. 1. Growth of A) preantral and B) large preantral follicles in the presence (triangles) or absence (circles) of IGF-1. Values are mean ± SEM. Growth is significant between Days 0 and 6 within both treatment groups ($P < 0.01$).

groups. Oocyte diameters are also represented here graphically using a box and whisker plot. The numbers of follicular granulosa layers at the end of culture were compared between groups using a chi-square test. With probability estimates for the occurrence of MMPs and TIMPs having been calculated, Baye's Theorem [23] was applied to estimate the probability of granulosa or theca cell health given that MMPs and TIMPs were present.

RESULTS

Follicle Growth

Follicles were cultured for 6 days in the presence (n = 72) or absence (n = 61) of IGF-1, with follicular diameters being measured every second day. For analysis of growth, follicles were separated into two size classes: preantral (<150 μm; n = 60), and large preantral (150–200 μm; n = 73). As Figure 1 illustrates, significant follicular growth occurred over 6 days in the presence and the absence of IGF-1 in both preantral and large preantral follicles ($P < 0.01$). No significant effect of IGF-1 on follicular growth was found in either size group ($P > 0.05$).

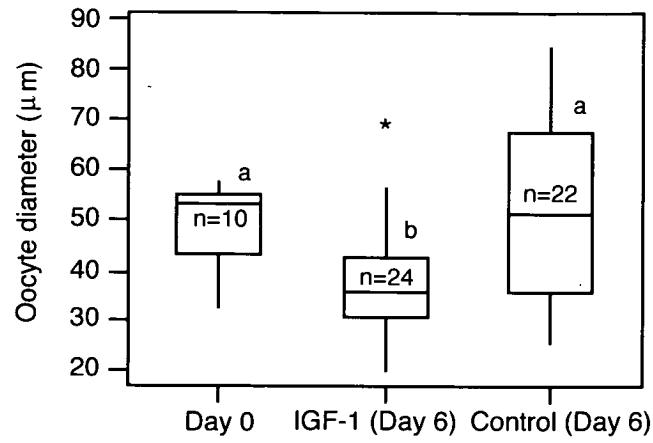

FIG. 2. Box and whisker plot of histological measurements of mean oocyte size on Days 0 and 6 of culture. n represents the total number of follicles analyzed for each treatment. Box represents the median and the upper and lower quartiles. Whiskers illustrate the expected data range (* denotes outlier). Different letters indicate significant differences ($P < 0.01$).

Histological Assessment

Histological observations were made using freshly isolated follicles (n = 10), IGF-1-treated follicles (n = 24), and control follicles (n = 22), all of which were 150–200 μm before culture. A significant difference in oocyte diameter between the three groups was detected by ANOVA ($P < 0.01$), and follicles treated with IGF-1 had significantly smaller oocytes than controls and freshly isolated follicles ($P < 0.01$) (Fig. 2). Follicles cultured with IGF-1 also had a reduced number of granulosa cell layers ($P < 0.01$) by Day 6 of culture (Table 1). However, IGF-1 had no effect on the percentage of follicles with degenerating oocytes and granulosa cells, the thickness and level of pyknosis of the follicular theca cell layers, or the percentage of follicles that formed an early antrum in vitro (Table 1). Examples of follicles with healthy and degenerating somatic cells are shown in Figure 3.

Secretion of MMP-2, MMP-9, TIMP-1, and TIMP-2 Activities

Culture medium from 32 follicles was analyzed for gelatinase activity, and 24 samples were analyzed for TIMPs. Examples of a zymogram demonstrating MMP-2 and MMP-9 activity and of a reverse zymogram showing TIMP-1 and TIMP-2 secretion by follicles into the culture medium are shown in Figure 4. The MMP-2 (72 kDa) was released by 87.5% of follicles, whereas the MMP-9 (92 kDa) was secreted by 62.5% of follicles (Fig. 4). Comparisons between zymographic analysis and morphology showed that if MMP-9 was secreted during culture, the probability of these follicles having healthy granulosa cells at the end of the culture period was 0.85. The probability of theca cell health (as defined by the number of

TABLE 1. Histological measurements of Day 6 of culture.

Treatment	Mean oocyte size (μm ± SEM)[a]	Mean no. granulosa layers (± SEM)	Mean no. theca layers (± SEM)	Oocyte degeneration (% follicles)	Granulosa degeneration (% follicles)	Theca degeneration (% follicles)	Antrum formation (% follicles)
Control[b]	52.4 ± 3.60	9.3 ± 0.41	3.9 ± 0.53	18.2	18.2	47.4	40.1
IGF-1[c]	37.8 ± 2.34[d]	6.4 ± 0.30[d]	3.0 ± 0.44	37.5	16.7	58.8	33.3

[a] SEM = standard error of the mean.
[b] n = 22.
[c] n = 24.
[d] Significantly lower than control ($P < 0.01$).

layers) was 0.60. When TIMP-1 (28 kDa) was released in vitro, (58% of follicles), the probability that the follicles would have healthy granulosa or theca cells was 0.79. When TIMP-2 (21 kDa) was produced (75% of follicles), the probability of the follicles having healthy granulosa or theca cells was 0.78 and 0.67, respectively.

DISCUSSION

The development of bovine preantral follicles to the early antral stage in a serum-free culture system was achieved, and secretion of MMPs and TIMPs correlated with follicular health during a 6-day period. We also demonstrated, to our knowledge for the first time, that IGF-1 has a negative effect on oocyte size and granulosa cell proliferation during this early stage of follicular development.

The IGF system has been well characterized in large antral follicles, but fewer studies have examined the effects of IGFs during earlier stages of follicular development. Wandji et al. [24] detected low IGF-1 mRNA levels in primary follicles from immature mice, but transcription increased to a maximum during the late preantral and early antral stages. In addition, apoptotic follicles had lower levels of IGF-1. This indicates that IGF-1 is associated with the growth and survival of rapidly expanding, large preantral and early antral follicles in the mouse [24, 25]. However, differences in the temporal and spatial production of IGF-1 between rodents and domestic species suggests different mechanisms of action. In sheep, the influence of IGF-1 on granulosa cells from small (1–3 mm) sheep follicles is proliferative, whereas in more mature follicles (5–7 mm), steroidogenic effects predominate [26]. Regarding the source of IGF-1, mRNA has been detected during the early

FIG. 3. Histological sections representing **A**) a follicle with a healthy oocyte (o), even granulosa cells (g) with spacing that may indicate an early antrum (ea), and a differentiated theca layer (t); and **B**) a follicle with pyknotic granulosa cells (p) and degenerated theca cells. Bar = 50 μm.

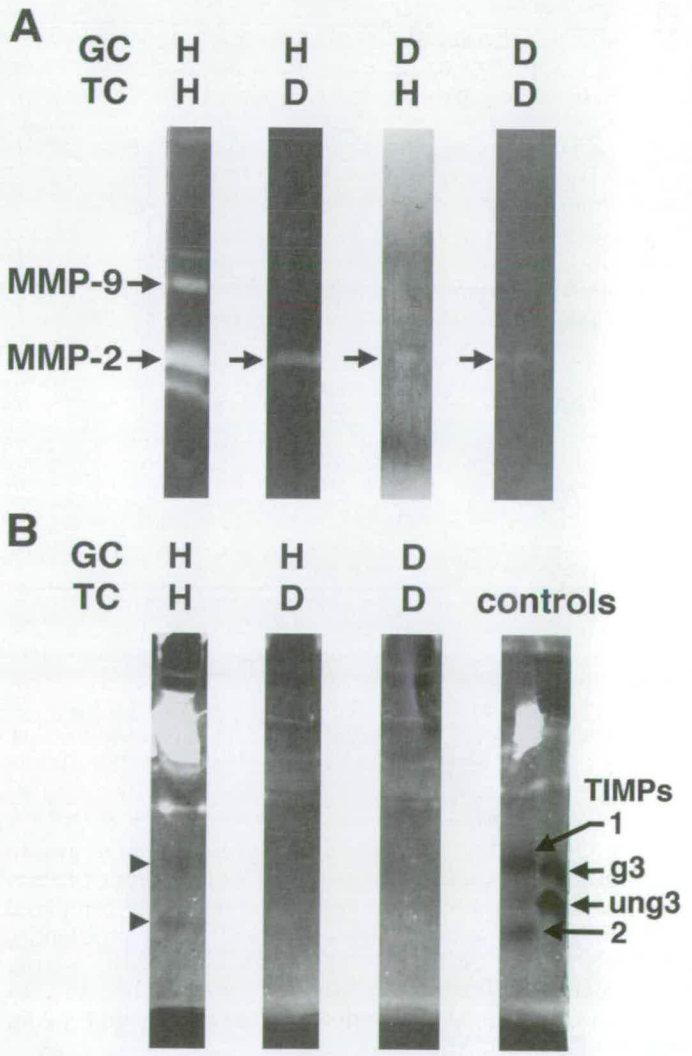

FIG. 4. Representative gelatin zymogram (**A**) and reverse zymogram (**B**) showing gelatinase (MMP) and TIMP activities secreted in samples of culture medium. The MMP-2 and MMP-9 or TIMP-1 and TIMP-2 presence is indicated in four follicles with granulosa cells (GC) that are healthy (H) or degenerate (D) and theca cells (TC) that are healthy (H) or degenerate (D).

antral stage in both granulosa and theca cells of sheep follicles [27] and in granulosa cells of porcine follicles [28], but no IGF-1 expression has been found during the preantral stage. In cattle, IGF-1 mRNA has been detected at low levels in granulosa cells of antral follicles, with an increased level after selection [29], and also in theca cells [30], indicating that IGF-1 may be important during the later stages of folliculogenesis (e.g., in relation to LH responsiveness).

Although IGF-1 attenuates spontaneous apoptosis in porcine granulosa cell cultures [31], it is not produced by ovine or bovine granulosa cells in vitro in the absence of luteinization [32, 33]. Receptors for IGF-1 on bovine granulosa cells do not fully develop until the antral stage [34], and this indicates a paracrine rather than an autocrine role for IGF-1 in the control of granulosa cell function, with IGF-1 playing a role later in the follicular cycle among domestic species than among rodents. Therefore, any positive effects on follicular development should be limited, which we have confirmed in the present study. Previously, Gutierrez et al. [11] reported that IGF-1 had a stimulatory effect on follicular and oocyte growth and antrum formation in an extended culture using confocal microscopy. They did not, however, present any data on follicular health. In addition, their study had limitations regarding the detection of an early antral cavity or early signs of atresia. Therefore, in the present study, we used histological analysis and a shorter culture period to detect the effects of IGF-1 during the early stages of development in more detail. A longer culture period may allow granulosa cells to differentiate sufficiently for IGF-1 to function as a stimulator of development, as reported by Gutierrez et al. [11]. Thus, the present study is novel, in that its results suggest the action of IGF-1 is strictly regulated according to developmental stage, and that treatment of immature follicles with IGF-1 may result in precocious differentiation, retarding growth and proliferation.

Regarding the preantral and early antral stages of follicular development, very little is known concerning the contribution of the various MMPs and associated regulators to follicular remodeling. We demonstrated that when MMP-9 and TIMPs are secreted in vitro, a follicle has a higher probability of being healthy at the end of the culture period. Several studies have investigated the distribution of MMPs and TIMPs at different stages of follicular development. In the ovary of neonatal rats, activity of MMP-2, but not MMP-9, has been detected, with visualization of TIMP-1 in the oocyte [20]. In eCG-primed ovaries of rats and mice, MMP-2 is detected in the granulosa and theca cells, MMP-9 is restricted to thecal and interstitial cells, and TIMP-1 is located in the blood vessels and theca cells [20, 35, 36]. In addition, MMP-9 is restricted to the theca cells layers in goat follicles of less than 3 mm [37], and it has been detected in the differentiating granulosa cells in culture [18] and, subsequently, in the developing corpus luteum of rats [20]. Collectively, the results of these studies implicate the granulosa and theca cells as sources of MMPs and TIMPs during the follicular stage of development.

In vitro, secretion of MMP-2 and MMP-9 from bovine thecal cells increases in response to LH [17]. This observation may imply that theca cells have a role in remodeling of the basement membrane, or that the remodeling of vascular tissue present within the culture may be under gonadotropin control. The regulation of MMP activity is complex and may be controlled at the level of transcription by growth factors, cytokines, and hormones, with subsequen activation of secreted proforms and inhibition by TIMP [36]. The mechanisms controlling remodeling at the gonad otropin-independent stages of follicular development are unknown; thus, further investigations are required. The MMPs and TIMPs also may be regulated depending on their specific spatial and temporal functions. The distribution of MMP-9 in the steroidogenic cells of the theca layer [20], interstitium, and corpus luteum as well as its absence in the neonatal ovary [20] and the timing of expression [38 suggest a role in the remodeling and vascularization associated with corpus luteum formation. The detection of MMP-2 and MMP-9 during our study in cattle suggests a role for these enzymes at a much earlier stage than, to our knowledge, has been studied previously, and that MMP-9 and TIMPs can now be used as noninvasive markers for assessing follicular quality in vitro.

The TIMPs have been implicated in other processes involving cell growth. For example, TIMP-1 has been associated with erythroid-potentiating activity [39] and TIMP-1 and -2 have growth factor-like activities in some cell types [40]. The increased probability of follicular health when TIMPs were present in our study agrees with this function. Results of other reports suggest that TIMP-1 is a facilitator of steroidogenesis [41–43]. Differences in TIMP activity between atretic and nonatretic follicles during the late preantral and early antral stage may be a marker for the shift in steroidogenic capability and future selection of healthy follicles at this stage of development. The significance of the location of TIMP-1 in the oocyte [20] is unclear, and during the early stages of folliculogenesis, a potential function for TIMPs as oocyte growth factors remains uninvestigated. Future experiments should assess if the correlation between MMP and TIMP secretion with follicular quality in vitro also applies to oocyte health and development. This will allow us to better understand interactions between the oocyte and somatic cells regarding the coordination of oogenesis and folliculogenesis.

In our study, follicles that did not maintain a differentiated thecal layer in vitro tended to be less healthy overall than follicles with thick, nonpyknotic theca cell layers. Maintaining interplay between the oocyte and the surrounding somatic cells is vital to achieve normal folliculogenesis. Growth differentiation factor-9 (GDF-9), which is an oocyte-derived factor from the transforming growth factor-β superfamily, is required for sustaining follicular growth and differentiation after the primary (i.e., one-layer) follicular stage [44]. Studies using knockout mice [44 45] have shown that in the absence of GDF-9, follicles are incompetent to emit a signal that recruits theca cell precursors to surround the basement membrane [45]. Our observations confirm the importance of maintaining healthy theca cells and their connections with the granulosa layers in bovine follicular culture, and our observations also support a role for these cell types in basement membrane remodeling during the early stages of folliculogenesis.

In conclusion, we have reported bovine follicular development from the preantral to the antral stage in a serum-free culture system. Use of IGF-1 as a treatment had no positive effects on follicular development during the preantral and early antral stages. We detected MMP and TIMP activity in vitro and identified MMP-9, TIMP-1, and TIMP-2 as markers of follicular health. Elucidation regarding the action of MMPs and TIMPs and the role o

growth factors during follicle growth and antrum formation in an extended culture will provide insight into the complex processes involved in follicular and oocyte growth and development. Improving our knowledge of the interactions between follicular cells and the extracellular matrix is necessary for maintaining the integrity of preantral follicles in vitro, thus sustaining follicular growth and viability.

ACKNOWLEDGMENTS

The authors thank Mr. D. Thomas for advice on the statistical analysis and Mr. J. Binnie for help with the histological preparation.

REFERENCES

1. Gosden RG, Telfer E. Scaling of follicular sizes in mammalian ovaries. J Zool (Lond) 1987; 211:157–168.
2. Ireland JJ. Control of follicular growth and development. J Reprod Fertil 1987; 34(suppl):39–54.
3. Van den Hurk R, Bevers MM, Beckers JF. In vivo and in vitro development of preantral follicles. Theriogenology 1997; 47:73–82.
4. Roy SK, Greenwald GS. Hormonal requirements for the growth and differentiation of hamster preantral follicles in long term culture. J Reprod Fertil 1989; 87:103–114.
5. Torrance C, Telfer EE, Gosden RG. Quantitative study of the development of isolated mouse preantral follicles in collagen gel culture. J Reprod Fertil 1989; 87:367–374.
6. Eppig JJ, Schroeder AC. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation and fertilisation in vitro. Biol Reprod 1989; 41:268–276.
7. Spears N, Boland NI, Murray AA, Gosden RG. Mouse oocytes derived from in vitro grown primary ovarian follicles are fertile. Biol Reprod 1994; 48:798–806.
8. Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. Biol Reprod 1996; 54:197–207.
9. Hirao Y, Nagai T, Kubo M, Miyano T, Miyake M, Kato S. In vitro growth and maturation in pig oocytes. J Reprod Fertil 1994; 100:333–339.
10. Cecconi S, Barboni B, Coccia M, Mattioli M. In vitro development of sheep preantral follicles. Biol Reprod 1999; 60:594–601.
11. Gutierrez CG, Ralph JH, Telfer EE, Wilmut I, Webb R. Bovine preantral to antral follicles after long term culture in vitro. Biol Reprod 2000; (in press).
12. Hulshof SCJ, Figueiredo JR, Beckers JF, Bevers MM, Van der Donk JA, Van den Hurk R. Effects of recombinant human FSH, 17β-estradiol and their combination on bovine preantral follicles in vitro. Theriogenology 1995; 44:217–226.
13. Ralph JH, Telfer EE, Wilmut I. In vitro growth of bovine preantral follicles and the influence of FSH on follicular and oocyte diameters. J Reprod Fertil Abstr Ser 1995; 15:6 (abstract 12).
14. Figueiredo JR, Hulshof SCJ, Thiry M, Van den Hurk R, Bevers MM, Nusgens B, Beckers JF. Extracellular matrix proteins and basement membrane: identification in bovine ovaries and significance for the attachment of cultured preantral follicles. Theriogenology 1995; 43: 845–858.
15. Giudice LC. Insulin-like growth factors and ovarian development. Endocr Rev 1992; 13:641–669.
16. Gutierrez CG, Campbell BK, Webb R. Development of a long term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to FSH and morphological characteristics. Biol Reprod 1997; 56:608–616.
17. Smith MF, Gutierrez CG, Armstrong DG, Webb R. Effect of LH on secretion of MMP by bovine thecal cells. J Reprod Fertil Abstr Ser 1997; 19:63 (abstract 169).
18. Zhao Y, Luck MR. Bovine granulosa cells express ECM proteins and their regulators during luteinization in culture. Reprod Fertil Dev 1996; 8:259–226.
19. Smith GW, Goetz TL, Anthony RV, Smith MF. Molecular cloning of an ovine ovarian tissue inhibitor of metalloproteinases: ontogeny of mRNA expression and in situ localisation within preovulatory follicles and luteal tissue. Endocrinology 1994; 134:344–352.
20. Bagavandoss P. Differential distribution of gelatinases and tissue inhibitor of metalloproteinase-1 in the rat ovary. J Endocrinol 1998; 158:221–228.
21. Byskov AGS. Cell kinetic studies of follicular atresia in the mouse ovary. J Reprod Fertil 1974; 37:277–285.
22. Riley SC, Leask R, Chard T, Wathen NC, Calder A, Howe DC. Secretion of matrix metalloproteinase-2, matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases into the intrauterine compartments during early pregnancy. Mol Hum Reprod 1999; 5:376–381.
23. Clarke GM, Cooke D. A basic course in statistics. London: Edward Arnold; 1992: 92.
24. Wandji SA, Wood TL, Crawford J, Levison SW, Hammond JM. Expression of mouse ovarian insulin-like growth factor system components during follicular development and atresia. Endocrinology 1998; 139:5205–5214.
25. Adashi EY, Resnick CE, Payne DW, Rosenfeld RG, Matsumoto T, Hunter MK, Gargosky SE, Zhou J, Bondy CA. The mouse intraovarian insulin-like growth factor system: departures from the rat paradigm. Endocrinology 1997; 138:3881–3890.
26. Monniaux D, Pisselet C. Control of proliferation and differentiation in ovine granulosa cells by insulin-like growth factor-1 and follicle stimulating hormone in vitro. Biol Reprod 1992; 46:109–119.
27. Leeuwenberg BR, Hurst PR, McNatty KP. Expression of IGF-1 messenger-RNA in the ovine ovary. J Med Endocrinol 1995; 15:251–258.
28. Yuan W, Lucy MC, Smith MF. Messenger ribonucleic acid for insulin-like growth factors-I and -II, insulin-like growth factor-binding protein-2, gonadotropin receptors, and steroidogenic enzymes in porcine follicles. Biol Reprod 1996; 55:1045–1054.
29. Schams D, Berisha B, Kosmann M, Einspanier R, Amselgruber WM. Possible role of growth hormone, IGFs, and IGF-binding proteins in the regulation of ovarian function in large farm animals. Domest Anim Endocrinol 1999; 17:279–285.
30. Gutierrez CG, Armstrong DG, Campbell BK, Hogg CO, Webb R. Insulin-like growth factor (IGF) I production and expression of IGF-I and II by bovine granulosa and theca cells in vivo and in vitro. J Reprod Fertil Abstr Ser 1996; 17:25 (abstract 66).
31. Guthrie HD, Garrett WM, Cooper BS. Follicle stimulating hormone and insulin-like growth factor-1 attenuate apoptosis in cultured porcine granulosa cells. Biol Reprod 1998; 58:390–396.
32. Wathes DC, Perks CM, Davis AJ, Denning-Kendall PA. Regulation of insulin-like growth factor-1 and progesterone synthesis by insulin and growth hormone in the ovine ovary. Biol Reprod 1995; 53:882–889.
33. Gutierrez CG, Campbell BK, Armstrong DG, Webb R. Insulin-like growth factor-1 (IGF-1) production by bovine granulosa cells in vitro and peripheral IGF-1 measurement in cattle serum: an evaluation of IGF-binding protein extraction protocols. J Endocrinol 1997; 153: 231–240.
34. Wandji SA, Pelletier G, Sirard MA. Ontogeny and cellular localisation of ^{125}I-labelled insulin-like growth factor-I, ^{125}I-labelled follicle-stimulating hormone, and ^{125}I-labelled human chorionic gonadotropin binding sites in ovaries from bovine fetuses and neonatal calves. Biol Reprod 1992; 47:814–822.
35. Reich R, Daphna-Iken D, Chun SY, Popliker M, Slager R, Adelmann-Grill BC, Tsafriri A. Preovulatory changes in ovarian expression of collagenases and tissue metalloproteinase inhibitor mRNA: role of eicosanoids. Endocrinology 1991; 129:1869–1891.
36. Hagglund AC, Ny A, Leonardsson G, Ny T. Regulation and localisation of matrix metalloproteinases in the mouse ovary during gonadotropin-induced ovulation. Endocrinology 1999; 140:4351–4358.
37. Garcia R, Ballesteros LM, Hernandez-Perez O, Rosales AM, Espinosa R, Soto H, Diaz de Leon L, Rosado A. Metalloproteinase activity during growth, maturation and atresia in the ovarian follicles of the goat. Anim Reprod Sci 1997; 47:211–218.
38. Chaffin CL, Stouffer RL. Expression of matrix metalloproteinases and their tissue inhibitor messenger ribonucleic acids in macaque periovulatory granulosa cells: time course and steroid regulation. Biol Reprod 1999; 61:14–21.
39. Docherty AJ, Lyons A, Smith BJ, Wright EM, Stephens PE, Harris TJR, Murphy G, Reynolds JJ. Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. Nature 1985; 318:66–69.
40. Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A. Cell growth promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). J Cell Sci 1994; 107:2373–2379.
41. Duncan WC, Illingworth PJ, Fraser HM. Expression of tissue inhibitor of metalloproteinases-1 in the primate ovary during induced luteal regression. J Endocrinol 1996; 151:203–213.
42. Nothnick WB, Curry TE. Divergent effects of interleukin-1 beta on

steroidogenesis and matrix metalloproteinase inhibitor expression and activity in cultured rat granulosa cells. Endocrinology 1996; 137: 3784–3790.
43. Boujrad N, Ogwuegbu SO, Garnier M, Lee C-H, Martin BM, Papadopoulos V. Identification of a stimulator of steroid hormone synthesis isolated from testis. Science 1995; 268:1609–1612.
44. Dong J, Albertini DF, Nishimori K, Rajendra Kumar T, Lu N, Matzuk M. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 1996; 383:531–535.
45. Elvin JA, Yan CN, Wang P, Nishimori K, Matzuk MM. Molecular characterisation of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 1999; 13:1018–1034.